BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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VOLUME XXXVI.

WOODS HOLE, MASS.

JANUARY TO JUNE, 1919

PRESS OF
THE NEW ERA PRINTING COMPANY
LANCASTER, PA.

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OF THE

Marine Biological Laboratory

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PUBLISHED MONTHLY BY THE
MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY
THE NEW ERA PRINTING COMPANY
LANCASTER, PA.

AGENT FOR GREAT BRITAIN
WILLIAM WESLEY & SON
28 Essex Street, Strand, London, W. C.

Single Numbers, 75 Cents. Per Volume (6 numbers), $3.00

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

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All communications and manuscripts should be sent to the Managing Editor. the University of Chicago, Sept. 15th to June 15th, or Woods Hole, Mass., June 15th to Sept. 15th. Subscriptions and other matter should be addressed to the Biological Bulletin, 41 North Queen Street, Lancaster, Pa

Vol. XXXVI. *January, 1919.* *No. 1*

BIOLOGICAL BULLETIN

THE FERTILIZATION REACTION IN ECHINARACHNIUS PARMA.

I. Cortical Response of the Egg to Insemination.

E. E. JUST.

Introduction.

So much has already been written about membrane "formation" in sea urchin eggs that one should perhaps hesitate to add to the list of papers on the subject. The observations here reported, however, would seem to fill a gap that has hitherto existed in a critical stage of the fertilization process. We know that the response of the egg to the spermatozoön on insemination first manifests itself in the appearance of a membrane at some distance from the vitellus—by some believed to be already on the uninseminated eggs; by others, to be actually formed *de novo*—but accounts as to why and how this membrane forms differ with different workers even where the same egg has been studied. Thus Herbst, Schücking, Kite and Heilbrunn among others have shown by different methods that the membrane is present on the unfertilized eggs; O. Hertwig originally at least expressed a similar view. R. Hertwig holds this view. On the other hand, Loeb, and the Loeb school generally not only believe that the membrane is formed after insemination but also that its formation is of great significance in the fertilization process; to Robertson, for example, membrane formation *is* fertilization. Again, we have the notion of Elder and of McClendon that the "fertilization membrane" is a precipitation membrane formed only in the presence of the intact jelly hull of the egg—a totally erroneous conception as shown by Harvey ('14). (See also, Lillie, '14.) Finally, while it is usually held that the membrane arises simultaneously from all points of the egg, suggestions have

not been wanting that the membrane forms as a wave that sweeps over the egg.

Fol first intimated that the membrane lifts from the egg as a wave dwelling on the rapidity with which the process is completed. Unfortunately he used eggs under pressure. Membrane formation as a progressive wave beginning at the site of sperm-entrance has been observed by Wilson who, however, is not at all sure. He says (in a footnote): "I have often observed that the formation of the membrane, in *Toxopneustes*, proceeds like a wave from the entrance-point around the periphery, but this is often irregular." Ries followed the fertilization with the cinematograph; he observed changes which indicated that the membrane forms first at one point.

In 1915 Dr. Tennent informed the writer that he had observed in cases a wavelike membrane formation. According to other workers, however, it would appear that no part of the cortex takes precedence in lifting off the membrane. Thus, Harvey ('10) who worked with *Toxopneustes*, *Hipponoë* and *Arbacia* says: "As observed in the living egg, almost immediately (1½ to 3 minutes) after addition of sperm the membrane substance becomes separated from the egg surface by spaces. These spaces fill with a fluid, unite and enlarge, thus pushing out the membrane some little distance.[1] This statement must refer to the egg of *Toxopneustes* (with which Wilson worked) or to that of *Hipponoë* for Heilbrunn after long experience with the egg of *Arbacia* was never able actually to follow the membrane elevation; he believed that in this egg the membrane lifted simultaneously from all parts of the cortex.

Loeb has followed membrane formation in the egg of *Strongylocentrotus purpuratus* by lowering the temperature of the seawater which retards the process. He gives eight figures of the process described. "The beginning of the process shows itself in a roughening of the hitherto smooth surface of the egg. This is due to the formation of countless tiny vesicles which stand out on the surface of the egg. These small droplets quickly increase

[1] The reader must not conclude from this statement that Harvey believes that the unfertilized egg possesses a membrane. In this paper he makes a categorical statement that the membrane arises at fertilization. In his 1914 paper he reaffirms this statement.

in size (through absorption of water) and flow together into larger drops. This goes on until finally the contents of all the drops have run together into a continuous layer around the egg. Hence the surface lamellæ of the tiny droplets form later the fertilization membrane." At higher temperatures, the egg of *Strongylocentrotus* forms a membrane very quickly passing directly from the condition of the uninseminated egg to that with fully formed membrane and wide perivitelline space; thus too in *Arbacia*. (Loeb, *loc. cit.*)

During a study of the fertilization reaction in the egg of *Echinarachnius* covering several seasons at the Marine Biological Laboratory at Woods Hole, Mass., I have made observations on membrane elevation in this egg where the process though it takes place with great rapidity can nevertheless because of the size of the egg be followed with remarkable ease. I can, therefore, make the unqualified statement that in the egg of *Echinarachnius* membrane elevation proceeds as a wave from the entrance-point of the sperm around the cortex. Moreover, what is more significant, before membrane elevation cortical changes blocking farther sperm entry spread as a wave over the egg uniting finally at the point opposite the entrance-point of the sperm.

Observations.[1]

If eggs of *Echinarachnius* be inseminated with thin sperm suspension they throw off membranes that are fully formed and equidistant from the surface of the vitellus in from two to three minutes. This follows sperm penetration which may take place at any point. Sperm is engulfed by the egg in from fourteen to forty-five seconds after insemination; the membrane begins to lift off in from seven to twenty-two seconds after sperm entry is complete; and membrane elevation is complete in from nine to thirty seconds after it begins. The shortest time recorded from insemination to membrane elevation from all parts of the egg is thirty-nine seconds, all time being taken with a stop-watch of a

[1] All observations were made on eggs in a quantity of sea-water; the eggs never suffered compression. Though jelly-free eggs form membranes, most observations were made on eggs with jelly so that the eggs were therefore free from flattening when on the bottom of the dish—the jelly acting as a buffer. Flattening of the eggs might easily lead to erroneous conclusions.

standard make. In some thirty to sixty seconds after the membrane is off from the egg it fully rounds out, equidistant from the surface of the cytoplasm at all points. The following tables taken from the data give some idea of the time relations. The time is in seconds. The last column ("Membrane Off") gives the time at which the membrane is distinctly off the egg at all points but not yet fully rounded. Each record is from observations of a single egg.

TABLE I.

Insemination.	Sperm Engulfed.	Membrane Begins.	Membrane Off.
0	50	65	80
0	49	64	84
0	30	50	70
0	45	67	
0	30	50	70

TABLE II.

Insemination.	Sperm In.	Membrane Begins.	Membrane Off.
0	30	45	57
0	15	37	53
0		44	66
0		32	57
0		29	39
0		37	48
0		38	47*

TABLE III.

Insemination.	Membrane Begins.	Membrane Off.
0	31	42
0	39	46
0	30	50
0	31	44
0	29	43
0	24	41
0	26	42
0	31	49
0	30	45
0	40	51
0	23	49
0	31	50
0	30	45
0	26	44
0	27	52
0	35	45
0	24	46

Average time of appearance of cone 90 seconds.
Average time of fully rounded membrane 134 seconds.

Immediately on insemination, sperm pierce the jelly hull, reaching the vitellus with rapid spiral movements; the moment the tip of the sperm touches the cortex of the vitellus all movements cease, the head and tail in a straight line at right angles to a tangent of the egg surface. Penetration follows as an activity of the egg; the spermatozoön does not bore its way in—the egg pulls it in.[2] After the head has disappeared within the cortex, membrane elevation begins.

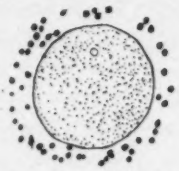


FIG. 1.[1] Freshly shed egg. Its pigmented jelly is as yet unswollen by the sea water.

After the sperm head has completely disappeared within the egg, the cortex reacts to penetration by pushing out a blister at the site of sperm entry (Fig. 2). This blister, which is not optically empty but contains minute drops that wander across this newly formed perivitelline space, may push off at once to a

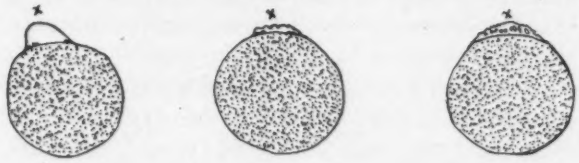


FIG. 2. An egg ten seconds after the disappearance of the sperm head within the egg.

FIG. 3. Same as Fig. 2.

FIG. 4. Four seconds after the membrane began lifting. Free vesicles beneath the membrane.

distance equal to the greatest the membrane ever reaches (Fig. 2); or, what is more common, the membrane elevation slowly sweeps (Figs. 3, 4, 5, 6, 7, 8) around the surface of the egg increasing the width of the perivitelline space after having lifted off from the egg at all points (Fig. 9). One may picture the process thus: By escape of substances from the cortex at the

[1] Figs. 1, 9, 10, and 11 were drawn with the aid of a camera lucida. All others are of necessity free-hand sketches. Except in Fig. 1, the jelly hull is omitted. x marks the site of sperm entry.

[2] Kupffer and Benecke in 1878 made similar observations on the lamprey egg and reached the conclusion that the sperm is engulfed.

point of sperm entry the membrane thoroughly glued to the egg surface is pushed off; the material continuing throughout the cortex to escape progressively lifts off the membrane around the egg.[1] The droplets are squeezed out of the cortex into the perivitelline space and may reach the membrane as discrete bodies

5 6

FIG. 5. About two seconds later than Fig. 4.
FIG. 6. Two to three seconds later than Fig. 5. Note vesicles forming.

before they fade from view. Often they go into solution more quickly. The membrane thickens slightly after formed.

As the membrane lifts off, it carries away any supernumerary sperm whose activity is in contrast to immobilized sperm previously engulfed by the egg. Not only is it true that at no

7 8

FIG. 7. About two seconds later than Fig. 6. Membrane incomplete at pole opposite entrance-point of the sperm.
FIG. 8. Twelve seconds after beginning of membrane lifting. The membrane is fully formed but still wrinkled, especially at point at which it lifted last.

point at which the membrane has elevated can sperm enter, but also that membrane elevation at a given point though not complete for the whole egg is a bar to sperm entrance at any point on the egg surface. And this is the most significant point in the reaction. *Before the actual elevation of the membrane, some cortical change beginning at the point of sperm entry sweeps over*

[1] This does not mean, however, that the eggs decrease in size after fertilization.

the egg immunizing it to other sperm; the direct opposite pole of the site of sperm entry is the last point affected.

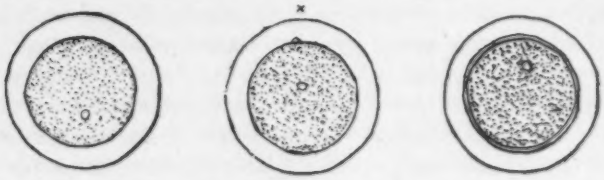
The site of sperm entry becomes a "point of injury" and is "negative" for any other sperm arriving at this point, all other portions around the egg being positive. This "wave of negativity" moves over the egg, its rate varying with the variations in the time that marks the disappearance of the sperm within the cytoplasm. When only the tip of the sperm head has entered the cytoplasm, the immediate vicinity of the site of penetration alone can not engulf sperm. As more of the sperm head disappears within the cortex of the egg, the "negativity" of the cortex for sperm entry progresses still farther around the egg until at the moment the head has disappeared the egg can engulf sperm only at one point—the pole opposite that at which the sperm entered the egg. A "wave of negativity" thus progressively sweeps over the egg from the point of sperm entry *preceding the actual beginning of membrane lifting.* Before the membrane begins lifting at the site of sperm entry sperm can no longer enter at any point on the egg.[1] From the point of sperm entry a definite gradient of membrane elevation is established, the last point of membrane elevation being at the pole opposite that of successful sperm entry. This gradient, therefore, follows that of diminished susceptibility to sperm penetration initiated at the entrance-place of the sperm.

If several spermatozoa become attached to the vitellus at the same instant membrane elevation starts at each point of attachment no matter what the distance separating them. Thus, two, three or more sperm may be attached at very nearly the same point or at varying distances from each other; in these cases other sperm later in arrival at the periphery of the egg, do not become engulfed, though they may be affixed by the egg. The membrane lifts at these points as blisters; each point behaves with reference to the space between it and an adjacent point of attachment as does the point in monospermic eggs, except that the "waves of negativity" spreading from each move to meet

[1] In 1878 O. Hertwig expressed the view that it is the egg plasma itself, if its vitality be unimpaired, which alone can prevent the entrance of more than one sperm.

thus diminishing the positive reaction of the cortex between two sperm points. Often, of course, even in heavy insemination a single spermatozoön reaches the egg at some one point ahead of others; in this case the egg is monospermic and so behaves. The observer is indeed struck with the rare occurrence of polyspermy even with heavy insemination.

When a spermatozoön becomes attached not only the penetration of other sperm but also their fixation to the vitellus depends upon the degree of penetration of the attached sperm or perhaps rather on the rate at which the "wave of negativity" is propagated around the egg. Thus, at the beginning of penetration, sperm nearby cannot become attached to the vitellus;



9 10 11

FIG. 9. Two minutes after insemination. Membrane is fully rounded, equidistant at all points from the now spherical egg.

FIG. 10. About same time as in Fig. 9. Cone is clearly visible.

FIG. 11. Ten minutes after insemination. Egg with hyaline plasma layer formed

those farther removed may become attached, lashing back and forth very vigorously. The heads of those still farther away but not far enough beyond the area of decrement actually to be engulfed are likewise affixed and present a swollen appearance. When the membrane lifts off these affixed sperm are carried with it.

The *Echinarachnius* egg, then, would seem to be unusual, since a gradient of susceptibility to the sperm may actually be demonstrated in the cortical changes following sperm entry.

After elevation of the membrane in *Echinarachnius* egg a cone (Fig. 10) of clear cytoplasm forms at the point of sperm entry; sectioned eggs show the sperm within where rotation of the head may begin. This cone[1] disappears; within some ten to fifteen

[1] The cone and the behavior of sperm that are attached to the cortex but do not enter constitute important checks on the observation of the point of sperm entry.

minutes after insemination a thin film forms on the surface of the cytoplasm (Fig. 11) the hyaline plasma layer, or gelatinous film of Loeb.

Experiments are being made at present with the hope of analyzing farther these cortical responses of the *Echinarachnius* egg to the sperm. We may, however, point out in conclusion to these observations that in the progressive membrane lifting and the cortical change that precedes it we have another of the numerous examples of an activity gradient; this of course is distinct from the primary axial (polar) gradient that exists in echinoderm eggs (see Child). In following these waves of cortical change we are actually viewing the propagation of the effect of a stimulus in an irritable cell. One effect of this stimulus of sperm entrance is to alter the plasma to such an extent that it prevents the entrance of other sperm. It is not the membrane that is a block to polyspermy; that block exists before the membrane lifts off. The membrane is merely the sign and consequence of more profound cortical changes.[1] In a way, it is of no significance that the membrane rises sharply into visibility provided these primary cortical changes take place. And indeed I have seen any number of eggs fertilize and develop without throwing off membranes. Such eggs are as incapable of reinsemination as eggs that have formed membranes. In the *Echinarachnius* egg, then, normal development has been already initiated by the sperm when the membrane begins to form.

[1] Says Lillie: "The fundamental mechanism for the prevention of polyspermy is the neutralization of the fertilizin by the anti-fertilizing present in the egg; *i. e.*, the occupancy of the spermophile side-chain of the fertilizin by the anti-fertilizin.

"The question may be raised why such neutralization of the fertilizin is delayed until the moment of fertilization? The answer to this difficulty is fairly clear. The fertilizin is located in the cortex of the egg, and the anti-fertilizin is more deeply situated; they therefore do not interact so long as the cell body as a whole is quiescent. But as soon as the cortical fertilizin becomes activated by union with the sperm it at once begins to attach certain substances in the egg, as demonstrated in the third part of this paper; this sets up diffusion evidenced by escape of pigment, and by cytoplasmic flowing, and the two substances are brought together and interact. While this explanation is partly hypothetical, the spatial separation of the fertilizin and anti-fertilizin and the quiescent character of the cell-body in the unfertilized egg are facts; so also are the movements of diffusion and the cytoplasmic currents set up on fertilization."

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THE FERTILIZATION REACTION IN ECHINARACHNIUS PARMA.

II. The Role of Fertilizin in Straight and Cross Fertilization.

E. E. JUST.

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I. Introduction.

The behavior of the sperm cells with reference to egg-extractives and the significance of this for the mechanism of fertilization has been analyzed in detail only in *Arbacia* and in *Nereis* (Lillie, '13, '14). It is true that the agglutination reaction had been previously studied by various workers, but the rôle of this phenomenon in fertilization as a specific reaction was not suggested; and it is on this specificity that the fertilizin theory of Lillie in large part rests; that is, that the egg alone secretes the sperm-agglutinating substance and that this substance is necessary for fertilization. It seemed desirable by study of this agglutinating reaction to determine its rôle in fertilization in some other form; this has been done in *Echinarachnius parma*. The present paper is a contribution to the analysis of the rôle of the sperm-agglutinating substance as revealed by a study of its behavior in straight and in cross fertilization. The results conform with those of Lillie, the data on cross fertilization seemingly at variance actually giving support to the view that the agglutination reaction is specific. The sperm agglutinin of *Echinarachnius* is, therefore, identical in behavior with the fertilizin of *Arbacia*.

Although observations on *Echinarachnius* were made during 1914, and 1915, all experiments here reported except those cited on page 22 are from those made, at the Marine Biological Laboratory at Woods Hole, Mass., during the season of 1917, beginning early in June. The experiments fall into two groups: those on iso-agglutination and straight fertilization in *Echinarachnius* (Part II.) and those on hetero-agglutination and cross fertilization with *Arbacia* (Part III.).

II. Sperm Agglutination and its Significance in Echinarachnius.

The mere fact that sperm are agglutinated by substances dissolved in sea-water is of no special significance for us since many substances will accomplish this; among others, alkalis, polyvalent salts (Gray), and snake venom (Noguchi); but the agglutinations by these agents are distinctly toxic and we must, therefore, at the outset exclude them from consideration. They

must in no wise be confused with agglutination produced by egg secretions. What is significant is that the agglutinin produced by eggs behaves in a characteristic fashion, may be quantitatively studied, and is a necessary part of the fertilization reaction.

A. *Sperm Agglutinin Production in Echinarachnius.*

Eggs of *Echinarachnius* charge sea-water with a substance that agglutinates *Echinarachnius* sperm. If a drop of this charged sea-water be added to a sperm suspension the sperm rapidly form clumps. The eggs give off this agglutinating substance in the absence of the jelly hulls enclosing them. Stale eggs give off relatively little and immature eggs none, of the agglutinin.

1. *Quantitative Method for Determining Agglutinin Production by Ova.*

By the method of successive dilutions of the sea-water taken from above *Echinarachnius* eggs after they have settled one may obtain quantitative estimates of the agglutinating power of the egg-secretion. This method, used by Lillie on *Arbacia*, briefly is as follows: A series of successive dilutions of the sea-water above *Echinarachnius* eggs is made, each dilution being one half the preceding. Usually I started with a 1/100 dilution of the agglutinin charged sea-water; a half dilution gives 1/200. Thus the series 1/100, 1/200, 1/400, 1/800, etc., is made. As noted below, series of 1/2, 1/4, 1/8, 1/16, etc., were likewise employed, so too, 1/10, 1/20, 1/40, etc. The agglutinating power of these dilutions is tested on sperm suspensions until the reaction comes negative. The last dilution to give a positive reaction rates the power of the agglutinin.

To observe the agglutination, drops of sperm suspension are mounted on a slide the cover slip of which is supported by glass rods, the slide being placed on the stage of the microscope in focus under low power. Into this sperm suspension, by means of a capillary pipette attached to a rubber tube, is blown a drop of the sea-water from the eggs. This microscopic method, as will be shown beyond, is indispensable for a comparative study of the agglutination reaction, or indeed for accurate quantitative determinations.

Agglutination may also be observed by adding two or three drops

of milky sperm suspension to two cubic centimeters of strong egg-water.[1] The sperm form clumps of such size that they are clearly visible to the naked eye, the sea-water between the masses remaining clear, so sharp is the reaction. This method suffices merely to indicate the *fact* of agglutination, and this only in high concentration. It was employed but seldom in these experiments as will be noted in the following pages. Indeed, by this method alone the critical points in this whole work would scarcely have been determined.

The methods for handling eggs and sperm are important since the state of the egg or sperm suspension conditions the experiment. To obtain eggs relatively free of debris, a mass of ovaries in two or three times their bulk of sea-water is strained through cheese cloth. Or, the animals may be rapidly opened and placed aboral side down in dry watch glasses and dry eggs collected as they exude from the genital pores. Also, eggs may be got as they are normally shed by animals in separate glasses. Sperm is collected from those males that have been opened, dried and placed aboral side down in dry watch glasses, and that soon shed sperm being pipetted off as needed. The sperm suspensions mentioned below, unless specifically noted to the contrary, were always one per cent. suspensions made by the addition of one drop of dry sperm to ninety-nine drops of sea-water. It is imperative to make up the suspension only as needed, for sperm reactions show wide variations depending upon freshness and upon concentration which likewise is a factor in ageing; the less concentrated suspensions deteriorate faster than more concentrated. Finally. everything was kept scrupulously clean, and all means taken to prevent contamination which might easily have invalidated whole series of experiments.

(*a*) *Variations in Fertilizin Production.*—Eggs vary in their capacity for producing fertilizin. In part this variation may be due to the fact that some suspensions do not consist wholly of eggs but may contain bits of tissue, etc.; such a suspension will be inferior to another of equal concentration but containing eggs only. However, this does not fully explain the variation, for it

[1] By egg water is meant sea-water taken from above eggs that have stood for a time.

is a simple matter by exercising care to procure, as mentioned above, from ovaries in sea-water clean egg suspensions entirely free from bits of tissue; these eggs as a class (which to avoid circumlocution I designate "ovary eggs") show wide variations and are inferior to dry or shed eggs, *i. e.*, those deposited by animals in clean dry dishes. Except for eggs mentioned on page (21) as normally resistant to fertilization, dry eggs produce more agglutinin than "ovary eggs" but show less variation.

The following observations from a large body of recorded data may now be cited.

1. July 11, 9:30 A.M.: To 1/2 c.c. of dry eggs sea-water was added to the 10-c.c. mark. At 10:10 7 c.c. of the supernatant sea-water was withdrawn and filtered. Tested with freshly prepared 1 per cent. *Echinarachnius* sperm, this withdrawn agglutinin charged sea-water was negative at 1/3,200 dilution and gave an 8 second reaction at 1/1,600.

2. July 11, 9:40 A.M.: Ovaries of 8 females in 20 c.c. of sea-water strained. Sea-water added to 100 c.c. Eggs settled at the 7 c.c. mark. At 10:20, 90 c.c. of supernatant sea-water decanted and filtered, which when tested on sperm came negative at 1/400 and gave a 6 second reaction at 1/200.

3. July 10, 10:30 A.M.: To 1.5 c.c. of dry eggs 8.5 c.c. of sea-water were added. 11:00 A.M. 8 c.c. of supernatant sea-water decanted and filtered, 8 c.c. being added to the eggs. 11:00 A.M. to 12:10 P.M., supernatant water of the *second washing* gave a 6-second reaction at 1/12,800 dilution.

Comparison of (1) and (2) shows that a 5 per cent. suspension of dry eggs yielded fertilizin of 1/1,600 power while a 7 per cent. suspension of ovary eggs contained fertilizin of but 1/200 power. Also, (3) shows us that after a *second washing* a 15 per cent. dry egg suspension gave fertilizin rated at 1/12,800 power. Without exception, the most powerful fertilizin suspensions were from shed eggs. These results are typical of a large number of observations.

(*b*) *Fertilizin Production by Washed Eggs.*—Lillie has shown that in *Arbacia* it is practically impossible by washing to remove all the fertilizin; as long as the egg lives it produces the agglutinating substance. The egg of *Echinarachnius* is not so hardy as that of *Arbacia;* becoming physiologically inert after remaining in sea-water for a time, it ceases to give off fertilizin though far from dead. This cessation is gradual, with each successive washing the fertilizin produced being less. Two experiments are cited.

1. July 2, 10:00 A.M.: Eggs from 4 females washed 5 times in 250 c.c. of sea-water at each washing. 2 c.c. of eggs after the fifth washing. At 12:00 M. 210 c.c. of supernatant water withdrawn and filtered, 210 c.c. of sea-water being added to the eggs. A sample of this removed at 1:30 P.M. on testing gave at 1/1,600 dilution a 10 second reaction. This is equal to morning tests. Washed 5 times during the afternoon by removing 210, 217, 203, 190, and 205 c.c. of water and adding in each case the quantity withdrawn. Tested at 8:30 P.M., supernatant sea-water gave an 8-second reaction at 1/1,600. July 3, 9:00 A.M. 210 c.c. of supernatant sea-water removed, 210 c.c. added. Tested during the day to 1:30 P.M. negative.

2. July 3, 9:10: Ovaries of 5 females in sea-water strained. Sea-water added to 100-c.c. mark. Washings (75 c.c. of water removed each time) as follows: 9:30, 9:34, 9:38, 10:05, 10:12 during the morning and at 1:50, 2:30 and 2:38 during the afternoon. Tested at 9:40, 10:50 and 1:30 gave 10 second reactions at 1/100. At 2:40, 8-second reaction on 1/50 dilution. 2:55 P.M. 75 c.c. of supernatant water removed, 75 c.c. added. 5 P.M., reaction faintly positive at 1/50. July 4, 10:30 A.M. 75 c.c. removed, 75 c.c. added. 11:10 A.M. negative when tested.

2. *Source of Fertilizin.*

In *Arbacia* only mature eggs produce fertilizin; immature eggs are incapable of secreting the agglutinating substance; other tissues do not elaborate it, nor does it occur in the perivisceral fluid (Lillie, '13, '14). In *Echinarachnius* mature eggs alone with or without jelly yield fertilizin to the sea-water. Neither the immature egg nor the body-fluid is capable of charging sea-water with sperm agglutinin.

(*a*) *Comparison of Ripe Eggs With and Without Jelly.*—Though the jelly is loaded with fertilizin, it does not form the fertilizin for jelly-free eggs are capable of charging the sea-water with the sperm agglutinating substance. The jelly hulls enclosing *Echinarachnius* eggs may be removed by shaking or by treatment with acid. The method of removing the jelly by shaking demands care since agitation may cause injury to the eggs. If shaking brings about the binding of fertilizin we should get a cessation of fertilizin production. This should not be attributed to mere loss of jelly, though it might be so interpreted by some. Again, it might be argued that the use of acid to remove the jelly might injure the egg; this objection could be raised only in the event that fertilizin is no longer produced by the egg after acid treatment, which is at least remotely possible. Certainly, it would be gratuitous to argue that if the fertilizin continues to be produced after acid treatment the acid carries the fertilizin from jelly to egg! In short, after shaking or after acid treatment

the cessation of fertilizin production would not prove that the jelly forms fertilizin. However, after both acid treatment and gentle shaking the eggs still produce fertilizin.

Echinarachnius eggs may be shed without jelly. Often in a given lot of eggs some will be found without jelly, and on three occasions during the summer I observed eggs shed *not one of which had a jelly covering*. These eggs proved to be normal in every respect; secreted fertilizin, were capable of fertilization, and of normal development.

I cite three experiments:

1. One c.c. of dry eggs was placed in 50 c.c. of sea-water plus 3 c.c. of *n*/10 HCl. Gently stirred to prevent clumping and washed in four changes of sea-water. Left in 50 c.c. of sea-water. Examination revealed absence of jelly in most eggs. Tested after one hour, the egg water gave an 8-second reaction at 1/200 dilution.

2. One c.c. of eggs was gently shaken five or six times in 10 c.c. of sea-water. Most of the eggs lost their jelly. The eggs were washed 5 times in 10 c.c. of sea-water and left finally in the cylinder in 10 c.c. of clean sea-water. On settling the eggs were at the 0.5-c.c. mark; tested, the supernatant sea-water gave at 1/400 dilution a 6-second reaction.

3. One c.c. of dry eggs shed without jelly was placed in 24 c.c. of sea-water. Tested an hour later the egg-water gave at 1/1,600 dilution a 6 second reaction. After a sixth washing in 3 hours these eggs produced agglutinin giving an 8-second reaction at 1/800 dilution.

Whatever may be the interpretation of the experiments with eggs freed of jelly through shaking or acid treatment, the conclusion drawn from the results with these eggs lacking jelly when shed is inescapable: in *Echinarachnius* as in *Arbacia* the fertilizin is secreted by the egg, however much the jelly may be loaded with it.

(*b*) *Study of Immature Eggs.*—During the season one may always obtain immature sand-dollars. Indeed one gets the impression that there is a rhythm in the breeding season. Loeb's observations ('15) would suggest that this is true of the California urchin, while Tennent[1] has noted the phenomenon which suggests lunar periodicity in the breeding of the Atlantic urchins. I was able, therefore, early in the season to determine quite definitely that immature *Echinarachnius* eggs do not give off fertilizin. Thus, on July 3, immature eggs from three females

[1] Dr. Tennent also tells me that in Greece the gonads of urchins used as food appear on the market only during certain moon phases; at other times the animals are spent.

were in turn tested. The supernatant sea-water from none during ten hours gave an agglutination reaction. Ovarian tissue, likewise, procured after removing the eggs by straining, does not produce a sperm agglutinin. The fully matured egg alone with or without jelly elaborates fertilizin.

(*c*) *Study of Perivisceral Fluid.*—Finally, repeated trials with with perivisceral fluid from mature or immature females never gave agglutination. Thus, at 2:40 P.M., July 3, drops of 1 per cent. *Echinarachnius* sperm suspension placed on slides when tested with perivisceral fluid from three females showed no agglutination. Each of the three samples of fluid was then diluted with clean sea-water 1/2, 1/4, 1/8, 1/16. The reaction with fresh sperm suspension in all cases was negative.

To sum up, we may say that mature *Echinarachnius* eggs in sea-water give off a substance that agglutinates *Echinarachnius* sperm; this substance the eggs will form in the absence of their jelly hulls. Immature eggs do not secrete the agglutinin nor does the ovarian tissue or perivisceral fluid.

The agglutinin of *Echinarachnius* is more powerful than that of *Arbacia*, as can be readily gleaned from a study of Lillie's work. An equal quantity of *Echinarachnius* eggs will give off more fertilizin in a given length of time than *Arbacia* eggs, but *Echinarachnius* eggs will cease production before *Arbacia* eggs. This is doubtless correlated with the greater irritability of *Echinarachnius* egg as evidenced both by its susceptibility to mechanical agitation and its earlier loss of fertilizing power.

It can be readily shown that this loss of fertilizing power is gradual and farther that it runs parallel with lessening fertilizin production.

B. *Fertilizin Production as an Index of Fertilization Capacity.*

While it is true that only mature eggs are capable of producing the agglutinin, this capacity varies between wide limits. One cannot foretell, therefore, the agglutinin production of a given lot of eggs in a given quantity of sea-water. Often eggs which on microscopic examination are found to be fully matured yield no agglutinating substance. Since, as shown above, immature eggs do not secrete fertilizin nor does the jelly form it, the secre-

tion must occur after the formation of the polar bodies. Fertilizin production, therefore, may be regarded as the sign of *physiological maturity*. This is borne out by a study of the parallel between fertilization capacity and fertilizin production. Eggs incapable of fertilization either have produced no fertilizin, unripe eggs, or have lost fertilizin, stale (washed) eggs.

1. *Ovary Eggs and Dry Eggs.*

As has been mentioned above, by cutting up ovaries in sea-water and straining the mass one may procure fairly clean egg suspensions free from bits of tissue. These are the "*ovary eggs.*" Dry or shed eggs are those deposited by the animals in clean dry dishes.

"*Ovary Eggs.*"—Different lots of "ovary eggs" vary greatly with respect to the agglutinin production, as repeated observations showed. A ten per cent. suspension made up of eggs from one female may give a positive reaction on 1/6,400 dilution while another ten per cent. suspension from another female may show little or even no agglutinative power. The capacity for fertilization corresponds so that following insemination ninety per cent. or more of the eggs with strong agglutination action cleave; very few of the eggs of no agglutinative action cleave. This is due to the fact that by cutting up the ovaries one obtains eggs of different degrees of ripeness. One lot from a given female may consist of fully ripened eggs while another lot contain scarcely any ripe eggs despite their appearance. If after washing the same pieces of ovaries several times, one collects eggs after each washing, the percentage of fertilization drops with each washing; the first lot of eggs is made up doubtless of those ready to be shed and so are fully ripened; successive lots are less mature. Moreover, dishes of "ovary eggs" usually contain some ovocytes. Without exception among these eggs those that fail to develop, give off no agglutinin; but those that have great capacity for fertilization show high agglutinating power.

"*Dry Eggs.*"—Dry or shed eggs are perfectly ripe, of high agglutination action, giving practically one hundred per cent. cleavage, beautiful large gastrulæ, and vigorous hardy plutei. The difference between these and "ovary eggs" is striking. Apparently when eggs are shed they are fully ready for fertiliza-

tion. Some years ago I observed similar behavior in *Asterias*. Eggs cut from ovaries may or may not fertilize: if they do cleave, subsequent development may be uncertain. Eggs naturally shed on the other hand, *may be inseminated at once*, fertilize easily, and develop perfectly, giving rise to larger and more vigorous larvæ. It would be a mistake to reach conclusions concerning the behavior of eggs and sperm unless these products have also been studied as they are normally shed. And I am disposed to believe that a great deal of the variability of eggs, so called physiological condition met with by workers, is due to the use of eggs not perfectly ripe. Dry eggs give powerful agglutinative suspensions.

2. *Washed Eggs.*

It is generally known that eggs lose their capacity for fertilization through remaining in sea-water, though the resistance to sea-water may vary within very wide limits depending upon the species of egg. Thus, the *Platynereis* egg can no longer be fertilized after six to ten seconds in sea-water, whereas the *Arbacia* egg may still be fertilized after several hours. In *Echinarachnius* the power to withstand sea-water varies with the kind of egg ("ovary egg" or dry), the time of season, the temperature, amount of mechanical shock, amount of sea-water used, etc. The most important factor is the condition of the egg since the most perfectly ripened eggs, *i. e.*, shed eggs, lose fertilization capacity earliest. There is an optimum period for fertilization and this gradually passes off as the eggs lie in sea-water. Eggs may lose their fertilization capacity in from two to fifteen hours; washing the eggs repeatedly will hasten this loss. Thus,

(*a*) 1 c.c. of eggs, samples of which when tested were fertilizable, was suspended in 99 c.c. of sea-water at 4:30 P.M., July 6. The supernatant sea-water gave on 1/800 dilution an 8-second reaction. Next day at 9 A.M. these eggs were unfertilizable before or after washing in 100 c.c. of sea-water. The superantant sea-water was negative to *Echinarachnius* sperm when tested throughout the day.

(*b*) The following is typical of a large number of experiments. On July 6 at 8 A.M., 2 c.c. of eggs were collected from three fine females washed in 5 c.c. of sea-water and strained. They were

washed seven times, agglutination tests and inseminations being made at each washing. The table gives the data.

EGGS FROM OVARIES OF THREE FEMALES.

Time.	Washings. No.	Agglutination Reaction.			Per Cent. Cleavage.
		C.c. of Sea-water Used.	Time in Seconds.	Dilution.	
8:30	1	100	8	1/1600	90
9:00	2	200	8	1/100	90
9:15	3	100	6	1/800	85
9:40	4	200	6	1/400	80
10:00	5	200	6	1/200	70
12:00	6	75	4	1/50	10
12:10	7	85	4		10

Began with 4 c.c. of eggs, ended with 2 c.c.

Two points are worthy of note respecting this table: First, the amount of water used in washing was unfortunately not the same throughout; and second, which is unavoidable, the quantity of eggs diminished with each washing. These two factors suffice to account for apparent irregularities in per cent. of cleavage and in agglutination. Thus, nos. 1 and 2 give the same per cent. of cleavage, whereas in no. 2 the agglutination reaction is but half as strong, but it is also true that twice as much sea-water was used in no. 2. The time between the washings is likewise irregular; this presumably would affect the amount of agglutinin present in the sea-water. However, the end result is unmistakable: low fertilization and low agglutination reaction are parallel. Since freshly made sperm suspensions were used for each insemination, the only other possible explanation is that the eggs die in large numbers. This is rather hazardous if not gratuitous since the eggs may make abortive attempts at development.

3. *Eggs Normally Resistant to Fertilization.*

On July 15 I procured 7 c.c. of dry eggs which were placed in 65 c.c. of sea-water. Also, by placing ovaries in sea-water for a few minutes before straining I obtained 3 c.c. of eggs which seemed to be unusually fine. Eggs from both lots were inseminated for preservation of a normal series. To my chagrin relatively few of these eggs segmented. July 16 gave similar results. Not until later did experiment reveal the cause of this failure to

segment. The observation of July 20 may serve as a typical case.

At 10:10 A.M., dry *Echinarachnius* eggs were placed in sea-water; total bulk of eggs plus sea-water equalling 10 c.c. (*Lot A.*)

10:20 A.M., 10 c.c. of ovaries plus 90 c.c. of sea-water were placed in a cylinder which was gently inverted three times. (*Lot B.*)

10:50 A.M., 7 c.c. of sea-water removed from eggs of *Lot A* filtered and diluted as follows: 1/10, 1/20, 1/40, 1/80, 1/160. 7 c.c. of fresh sea-water added to eggs. Trials with sperm of two males gave a 6-second reaction at 1/20.

11:20 A.M., 6 c.c. of supernatant sea-water removed and filtered, dilutions being made as before. (6 c.c. of fresh sea-water added to eggs.) 4–(?)-second reaction at 1/10 dilution; very faintly positive.

11:50 A.M., 5 c.c. of supernatant sea-water removed and filtered with dilutions as before, 6-second reaction at 1/10. Eggs inseminated 3:00. These normally fertilized eggs show very few cleavages. *Lot B* gave comparable results.

If this were a long series of washings extending over two or three days, one might suggest that these eggs no longer fertilize or give the agglutination test because they are dead. One could scarcely have the hardihood, though, to venture that the eggs die after remaining in sea-water some ninety minutes. Rather these eggs fail to fertilize, as was discovered during the next two days because of some membrane condition, for on the addition of ether to the sea-water, eggs, of which but 1 per cent. showed cleavage in normal sea-water, gave 90 per cent. cleavage and normal larvæ. I might designate these eggs as over-ripe following R. S. Lillie's usage since they behave so much like over-ripe *Asterias* eggs. The cleavage percentage of these eggs is always low and their agglutinin production feeble. Unfortunately, I failed to study the agglutinative power of these ether-treated eggs.

4. *Fertilizin Production After Membrane Formation.*

Observations on eggs with membranes—which were not made before the season of 1918—show that after insemination or successful butyric acid treatment fertilizin production ceases.

(*a*) *Eggs with Fertilization Membranes.*—Lillie found ('14, pp. 553–557) that in *Arbacia* fertilized eggs with jelly removed by shaking ceased to give the fertilizin test after five washings (extending over a period of one hour and forty-three minutes), the last washing being made at two hours and twenty-three minutes after insemination; while an equal quantity of eggs of

the same inseminated lot *with* jelly still gave a test of over seventy-five seconds. When these latter came negative is not stated. From this and duplicate experiments Lillie concluded that after fertilization the egg no longer produces fertilizin; the positive tests with jelly-covered eggs show merely that the jelly is saturated with the secretion. I have repeated this experiment with *Echinarachnius* eggs and have also made some others slightly different. My findings agree with those of Lillie.

An experiment of August 1 is typical of a number:

9:15 A.M. Two tubes (*A* and *B*) each with 0.5 c.c. of eggs from the same female plus 9.5 c.c. of sea-water; 9:16 A.M. inseminated; 9:18 A.M. all eggs have membranes; 9:20 A.M. tube *B* gently shaken to remove jelly; 9:23 A.M. jelly off every single egg, membranes intact. By means of a capillary pipette attached to a rubber tube supernatant sea-water carefully removed from each tube as follows: 9:35, 9:45, 10:00, 10:16, 10:31, 11:00 and 11:13 A.M.; in each case 8 c.c. being removed and 8 c.c. of fresh sea water added. At 11:05 A.M. at 1/1 dilution *B* is slightly positive (?); at 11:15 A.M. and thereafter *B* is negative at 1/1 dilution. *A* at 1/8 dilution gave an 8-second reaction. 99 + per cent. of the eggs in both lots developed normally.

Thus in two hours after insemination (seven washings) the tests on jelly-free eggs came absolutely negative. (*Vide supra*, Lillie's results.)

The data on some of these experiments seemed to indicate that the fertilizin is not lost in any given time but rather after a certain amount of washing. If this be true, a certain amount of water ought to remove from a given lot of eggs the free fertilizin practically at once. The following experiments show this:

Aug. 2, 9:40 A.M. Two cylinders (*A* and *B*) each with 0.5 c.c. of eggs from the same female plus 9.5 c.c. of sea water. Both inseminated. 9:43 A.M. *B* shaken. Eggs have 100 per cent. membranes in both *A* and *B;* none have jelly in *B*.

9:50 A.M. Lots *A* and *B* each in 250 c.c. of sea-water.

9:53 A.M. *A* is positive at 1/2 dilution; *B* is negative at 1/1 dilution, never again positive.

Aug. 3, 9:58 A.M. 2.5 c.c. of eggs from one female plus 7.5 c.c. of sea-water inseminated and divided into two equal lots—*A* and *B*. *B* shaken. *A* and *B* each added to 250 c.c. of sea-water. 10:05 A.M. *B* positive at 1/1 dilution. 10:07 A.M. 190 c.c. of sea-water removed from *A*, 200 c.c. of sea-water removed from *B*. 190 c.c. and 200 c.c. of sea-water added to *A* and *B* respectively. 10:25 A.M. *A* barely positive at 1/8 dilution; *B* is negative at 1/1.

These observations bear out findings of June: while making experiments with shaken eggs I tested fertilized jelly-free and membrane-free eggs and never obtained a positive test. Evi-

dently, Lillie's assumption "that all free fertilizin is fixed *at the moment of* fertilization or membrane formation" is correct.

(*b*) *Eggs with Butyric Acid Membranes.*—If eggs be successfully treated with butyric acid (optimum exposure to optimum concentration of butyric acid in sea-water) they form membranes. In highly successful cases one may obtain one hundred per cent. membranes. These eggs behave as do eggs with fertilization membranes. If washed by repeated removal of the supernatant sea-water they cease producing fertilizin within two hours after five or six washings. If eggs be washed by removal directly to a large quantity of sea-water the residual fertilizin (in the jelly which may not be removed by butyric acid) is washed away so that when tested these eggs are negative; while an equal bulk of unfertilized eggs *without* jelly suspended in a like amount of sea-water will still give the reaction.

We may conclude that after membrane formation induced by sperm or by butyric acid eggs no longer secrete fertilizin.

According to Lillie's studies, and likewise Moore's account ('16), fertilization capacity and fertilizin production in *Arbacia* run parallel. I have found the same to be true in *Platynereis* and *Nereis*. Farther, *Nereis* exhibits a very striking phenomenon for even slight washing renders impossible initiation of development by warming. In the eggs of *Echinarachnius* the measure of fertilizability is agglutinin production.

C. *Summary of Part II.*

We have shown (1) that *Echinarachnius* eggs in sea-water liberate a substance that agglutinates *Echinarachnius* sperm. This substance, fertilizin, which can be quantitatively studied is formed most abundantly in fully ripened (shed) eggs. The substance is lost by eggs repeatedly washed. It is formed by ripe eggs with or without jelly but is not formed by immature eggs nor is it found in perivisceral fluid. (2). Fertilizin production is an index of fertilization capacity. Eggs highest in fertilizin content, shed eggs, fertilize in largest numbers. Washing the eggs decreases both the fertilizin secretion and the percentage of fertilization. Eggs normally resistant to fertilization yield little fertilizin. After membrane formation fertilizin production ceases.

III. Cross Fertilization and Agglutination in *Echinarachnius* and *Arbacia*.

We have seen in Part II. that the eggs of *Echinarachnius* in sea-water liberate a substance comparable in every way to the substance, fertilizin, liberated by *Arbacia* eggs. I have, therefore, used the term fertilizin for the *Echinarachnius* substance. The problem that next presented itself was the study of cross-fertilization in these two echinids whose sperm behave in the same way to their egg secretions. In a relatively short time, a rather curious, but as it turned out merely apparent, contradiction was in evidence: The cross was highly successful in one direction only and the agglutination reaction ran counter to and not parallel with cross fertilization capacity. This seemed indeed worthy of study. A great deal of time, therefore, was devoted to repeating the initial attempts at cross fertilization and to determining the nature of, and the part played by, the hetero-agglutinating substance.[1]

In this section I shall present first the results of the cross fertilization and second some experiments which attempt to analyze the significance of the hetero-agglutination.

A. *Reciprocal Cross Fertilization of Echinarachnius and Arbacia.*

With various methods many echinid crosses have been obtained by a large number of workers; of these, the crosses by Tennent and by Vernon may be particularly mentioned because of the widely differing forms employed. The value of these methods is discussed by Tennent and need not be commented on here. Moreover, my object was not so much to test the relative value of these methods for *Arbacia-Echinarachnius* crosses as to test under the same conditions the response of the *Arbacia* egg and of the *Echinarachnius* egg to foreign sperm. Comparison of the capacity of the eggs for cross fertilization is made only where the same method was employed on both eggs. And I may say at once that while it is relatively easy to fertilize *Echinarachnius* eggs with *Arbacia* sperm, the reciprocal fertilization is extremely difficult.

[1] Abundant material of both straight and cross fertilizations was fixed in a number of fluids. The results of the study of this will appear later.

Three methods were used to obtain cross fertilization: allowing the eggs to stand, treating them with alkali, and subjecting them to concentrated sperm. In each experiment reciprocal crosses[3] were made, but it will be convenient to consider them separately. I shall begin with the *Arbacia* ♀ × *Echinarachnius* ♂.

1. *Arbacia* ♀ × *Echinarachnius* ♂.

1. *Arbacia eggs allowed to stand* in sea-water upward to thirty-six hours show little development following insemination with *Echinarachnius* sperm. For example, on July 2 I made an observation subsequently repeated throughout the season:

July 2. Eggs of *Arbacia* placed in a finger bowl of sea-water from which two samples were removed at hour intervals, from 8 A.M. to 10 P.M.; one sample inseminated with *Echinarachnius* sperm, the other uninseminated control. Hourly inseminations were continued during the next day until 4:00 P.M., a final insemination at 6:00 P.M. One lot, 24-hour, showed 1 per cent. cleavage, its control about 0.1 per cent.

Results with shed eggs placed in sea-water without subsequent washing and with eggs thoroughly washed by changing the water repeatedly show slight differences.

2. *With alkali treatment* the results are practically negative for the amounts of alkali used; whenever development followed the per cent. was extremely low.

July 7, 5:00 P.M. Eggs in 10 c.c. of sea-water plus 2 drops of *n*/10 KOH were inseminated with *Echinarachnius* sperm. 6:40 P.M. Not a single egg formed a membrane. Later none of these developed.

3. *Treatment with concentrated sperm* gave the best results between one and three per cent. of eggs developing. For example, I quote from a long experiment of July 9.

5:00 PM. Five drops of dry eggs from one *Arbacia* were put in each of four watch glasses *A*, *B*, *C*, *D*. To *A*, one drop of *Echinarachnius* sperm suspension added and 5 c.c. of sea-water; to *B*, one drop of *Echinarachnius* sperm suspension and after five minutes 5 c.c. of sea-water; to *C Arbacia* sperm and 5 c.c. of sea-water; *D*, uninseminated control. *A* gave 1 per cent. cleavage; *B* 2 per cent.; *C*, 95 per cent.; and *D* not *one membrane or a cleavage.*

Washing the eggs thoroughly would seem to be beneficial to treatment with concentrated sperm but the data on this I deem at present insufficient to be conclusive.

On the whole, the cross, *Arbacia* ♀ × *Echinarachnius* ♂ *is*

not highly successful; often none of the methods gave results. The reciprocal is not so difficult.

2. *Echinarachnius* ♀ × *Arbacia* ♂.

The cross *Echinarachnius* ♀ × *Arbacia* ♂ as well as the reciprocal cross was made usually during agglutination experiments. Thus, after removing the supernatant sea-water from eggs for agglutination tests these could be used for straight or cross fertilization. This method, in addition to being economical, made possible the performing of a large number of experiments during the time the *Echinarachnius* eggs are at their best.

1. *Allowing the Eggs to Stand.*—Although as mentioned above, *Echinarachnius* eggs are not so resistant to sea-water as *Arbacia* eggs, after three to four hours in sea-water they develop following insemination with *Arbacia* sperm. Thus on July 4, *Echinarachnius* eggs were inseminated with *Arbacia* sperm at ten-minute intervals up to three hours. The best lot of eggs was that inseminated after two hours in sea-water and showed five per cent. of development. Addition of *Arbacia* egg-water to the eggs does not increase the per cent. of those that develop.

2. *Treating the Eggs with Alkali.*—Treatment with alkali before and after insemination gives a high per cent. of cleavage; the eggs, however, in the later stages, particularly the blastula and gastrula, show marked abnormalities as well as a tendency to disintegrate.

On July 7, the following experiment was made three times:

2:40 P.M. Eggs previously procured by straining from ovaries gently cut up in sea-water were divided among seven dishes:

No. 1, uninseminated control in 15 c.c. of sea-water.

No. 2, eggs + 15 c.c. sea-water + 1 drop *n*/10 KOH.

No. 3, eggs + 15 c.c. sea-water + 2 drops *n*/10 KOH.

No. 4, eggs + 15 c.c. sea-water + 3 drops *n*/10 KOH.

2:42 P.M. Nos. 2, 3 and 4 inseminated with freshly made thin *Arbacia* sperm suspension.

2:45 P.M. Nos. 5, 6 and 7. Eggs in 15 c.c. of sea-water inseminated with same *Arbacia* sperm.

2:47 P.M. 1, 2 and 3 drops *n*/10 KOH added to nos. 5, 6, and 7 respectively.

5:00 P.M. Eggs show five per cent. development; in No. 2, much less in 3, 4, 5, 6, 7. Control shows no development.

With shed eggs the results are strikingly different.

July 8, 3:10 P.M. To dry eggs from one female, 100 c.c. sea-water added.

3:20 P.M. To each of seven watch glasses a drop of eggs is added.

No. 1, uninseminated control in 15 c.c. of sea-water.

No. 2, Eggs + 15 c.c. of sea-water + 1 drop *n*/10 KOH.

No. 3, Eggs + 15 c.c. of sea-water + 2 drops *n*/10 KOH.

No. 4, Eggs + 15 c.c. of sea-water + 3 drops *n*/10 KOH.

3:22, Nos. 2–4 inseminated each with one drop of freshly prepared active *Arbacia* sperm suspension.

3:24. Nos. 5, 6, and 7, each with 15 c.c. of sea-water inseminated with one drop of same *Arbacia* sperm.

3:26. To nos. 5, 6 and 7 respectively are added 1, 2 and 3 drops of *n*/10 KOH.

5:00. These eggs show high per cent. cleavage (90 per cent. in no. 4), but also a tendency to lose their membranes when put in normal sea-water. Control, no development.

3. *Insemination with Excess of Sperm.*—The method of heavy insemination succeeds with *Echinarachnius* eggs, giving ten to twenty-five per cent. of cleavage. The later stages are far superior to those eggs in which crossing has been induced through alkali treatment.

July 3, 9:10 A.M. Eggs removed from ovaries divided into three lots—*A*, *B* and *C*. *Lot A* inseminated at 9:15 A.M. with heavy *Arbacia* sperm; *Lot B* similarly at 11:00 A.M.; and *Lot C* similarly at 2:55 P.M. *Lot A* shows 13 per cent. of development at 2:50. *Lot B* 8 per cent. *Lot C* at 5:00 P.M. shows no development.

July 11, 9:30 A.M. 1/2 c.c. of dry *Echinarachnius* eggs placed in 10 c.c. of sea-water. 10:05 A.M., five drops of eggs in each of four watch glasses (Series I.) containing 1, 2, 3 and 4 drops of freshly prepared *Arbacia* suspension. To each of four watch glasses (Series II.) containing five drops of eggs, 1, 2, 3 and 4 drops of *Arbacia* sperm added. Watch glass no. 9 uninseminated control; no. 10, inseminated with *Echinarachnius* sperm.

1:30 P.M. Uninseminated control, no development. Eggs inseminated with *Echinarachnius* sperm 98 per cent. developing. Eggs of both series inseminated with *Arbacia* sperm developing—25 per cent. in most dishes.

These experiments with heavy insemination show that the eggs develop best when inseminated soon after procured; also, that dry eggs are better than "ovary eggs." Whether eggs are added to sperm or vice versa is of no monent, but sperm must be fresh. The eggs treated with dense sperm suspension develop more nearly like the straight fertilized eggs than either the alkali treated or the stale eggs. The larvæ are vigorous and active, swimming to the top as do normal larvæ.[1]

The cross *Echinarachnius* ♀ × *Arbacia* ♂ succeeds far better than the *Arbacia* ♀ × *Echinarachnius* ♂.[2] Indeed in some cases the *Arbacia* egg is in no wise affected by *Echinarachnius*

[1] Larvæ from this cross were kept for four weeks.

[2] Cf. Gemmil's results with the cross, *Cribrella* × *Asterias*.

sperm. Thus, we have a sharply defined difference in behavior between the *Echinarachnius* egg and the *Arbacia* egg with respect to foreign sperm.

B. *Hetero-agglutination in Echinarachnius and Arbacia.*

If fertilizin is necessary for fertilization and if it is likewise specific, we should expect that where cross fertilization is possible in one direction only the sperm agglutination reaction would, if it occurred at all, take place in the same direction The opposite appears to be the case for the *Echinarachnius* × *Arbacia* cross since *Echinarachnius* egg-water has no effect on *Arbacia* sperm while *Arbacia* egg-water has a powerful agglutinating effect on *Echinarachnius* sperm. However, as shown beyond this is not the effect of *Arbacia* sperm agglutinin but is due to a separate substance, a hetero-agglutinin.

1. *Effect of Echinarachnius Egg-water on Arbacia Sperm.*

Sea-water from above *Echinarachnius* eggs highly charged with the substance that agglutinates *Echinarachnius* sperm has no agglutinative action on *Arbacia* sperm. This is true of the most powerful *Echinarachnius* egg-water that was used. The *Echinarachnius* egg-water does, however, intensely activate *Arbacia* sperm.

Experiments were made with egg-water of both "ovary" and dry eggs.

July 2, 10:00 A.M. Eggs from four *Echinarachnius* strained and washed 5 times in 250 c.c. of sea-water. 2 c.c. of eggs and small bits of ovary. 12:00 M., 210 c.c. of sea-water removed and filtered. Tests with *Echinarachnius* sperm, at 1:30 P.M. and 8:30 P.M., showed great agglutinating strength.

11:00 A.M. Eggs from *Arbacia* washed 5 times in 100 c.c. of sea-water at each washing. 12:05 P.M., 45 c.c. of supernatant sea-water removed and filtered. Tested with *Arbacia* sperm, showed very powerful reaction.

3:00 P.M. *Arbacia* egg-water agglutinates *Arbacia* sperm; *Echinarachnius* egg-water agglutinates *Echinarachnius* sperm, but fails to agglutinate *Arbacia* sperm—trials being made on sperm of three males.

July 10, 10:30 A.M. 1 1/2 c.c. of dry eggs of *Echinarachnius* in 8 1/2 c.c. of sea-water. 11:00 A.M. 8 c.c. of sea-water were pipetted off and filtered. (8 c.c. of fresh sea-water added to the eggs.) 11:05 A.M. 7 c.c. of *Arbacia* eggs from four females put in 50 c.c. of sea-water.

12:10 P.M. *Echinarachnius* secretion gives a 5–6-second reaction with its sperm on 1/500 dilution; full strength, this fails to agglutinate *Arbacia* sperm. *Arbacia* sperm suspensions used were 50 per cent., 20 per cent., 10 per cent. and 1 per cent. freshly prepared in each case. *Arbacia* egg-water agglutinates *Arbacia* sperm.

2:15 P.M. 8 c.c. of sea-water pipetted from the *Echinarachnius* eggs and filtered (8 c.c. of sea-water added to the eggs). 2:00 to 4:00 P.M. This egg-water removed (second washing) gave on *Echinarachnius* sperm a 16-second reaction at 1/12,800 dilution. It failed to agglutinate *Arbacia* sperm but activated them very intensely. *Arbacia* egg-water on these same sperm gave an 8-second reaction on 1/6,400 dilution.

July 16. A strong *Echinarachnius* egg-water, 1/12,800 power, tested on *Arbacia* sperm gave no agglutination, but very intense activation.

The experiments here cited represent but a small percentage of the total number made with *Echinarachnius* egg-water on *Arbacia* sperm. There can thus be no question as to the failure of the *Echinarachnius* egg-water to agglutinate *Arbacia* sperm. On the other hand, the stimulation to increased motility induced by *Echinarachnius* egg-water is very remarkable.[1] In no other case that I know is the activation so marked. It will be noted that the egg-water used is that charged either by thoroughly washed "ovary eggs" or by dry eggs; this, a fact which may be significant, must mean that such sea-water contains very little if any perivisceral fluid.

2. *Effect of Arbacia Secretion on Echinarachnius Sperm.*

Sea-water charged by *Arbacia* eggs with an agglutinating substance for *Arbacia* sperm likewise contains a substance that agglutinates *Echinarachnius* sperm. Certain characters of this agglutination of *Echinarachnius* sperm show that it is due to a hetero-agglutinin distinct from the *Arbacia* sperm agglutinin.

(*a*) *Agglutinative Power of Arbacia Egg-water on Echinarachnius Sperm after Successive Dilution.*—In the first place it may be noted that on dilution of *Arbacia* egg-water the agglutinating substance for *Echinarachnius* sperm drops out before the agglutinin for *Arbacia* sperm. A freshly prepared suspension made of unwashed "ovary eggs" may agglutinate *Echinarachnius* sperm in a very powerful manner, irreversibly so in some cases; but if the egg-water stand or be diluted this effect will gradually disappear, the iso-agglutinin remaining. On the other hand, a suspension of shed eggs not uncommonly gives no agglutinin for *Echinarachnius* sperm, though this class of eggs most actively secrete iso-agglutinin.

[1] *Asterias* sperm exhibit an interesting phenomenon. When a fresh suspension is prepared, the sperm may be absolutely immobile and remain thus. If now they be mixed with eggs inseminated within one or two minutes previously, they are stimulated to great activity so that they may roll the eggs around.

July 10, 11:05 A.M. 7 c.c. of *Arbacia* eggs from four females in 50 c.c. of sea-water. 12:15, 40 c.c. pipetted off and filtered (40 c.c. of fresh sea-water added to the eggs). 4:00 P.M. *Arbacia* egg-water (first washing) gave an 8-second reaction with *Arbacia* sperm at 1/12,800 dilution. This same suspension gave a 3–4-second reaction on *Echinarachnius* sperm at 1/800 dilution.

July 16, 11:00 A.M. 7 c.c. of *Arbacia* eggs and ovarian tissue put in 50 c.c. of sea-water; strained giving 55 c.c. 1:40 P.M. 37 c.c. of supernatant sea-water pipetted off and filtered, 80 c.c. of sea-water being added to eggs. 2:30 P.M. Supernatant sea-water withdrawn gave at 1/6,400 dilution a 6-second reaction with *Arbacia* sperm; faintly positive for *Echinarachnius* at 1/100 dilution.

(*b*) *Hetero-agglutinin Production of Washed Arbacia Eggs.*—In the second place, if *Arbacia* eggs be repeatedly washed the hetero-agglutinating substance drops out before the iso-agglutinin. A single washing may suffice to remove the hetero-agglutinin. Certainly washing during twelve hours is sufficient entirely to remove the hetero-agglutinin.

July 12, 6:30 P.M. *Arbacia* eggs in 250 c.c. of sea-water; eggs settle at the 7-c.c. mark. 140 c.c. poured off and 140 c.c. sea-water added.

7:30 P.M.—220 c.c. sea-water poured off, 220 c.c. added.
8:30 P.M.—190 c.c. sea-water poured off, 190 c.c. added.
10:00 P.M.—140 c.c. sea-water poured off, 140 c.c. added.
July 13.
8:15 A.M.—210 c.c. sea-water poured off, 210 c.c. added.
9:15 A.M.—150 c.c. sea-water poured off, 150 c.c. added.
10:10 A.M.—200 c.c. sea-water poured off, 200 c.c. added.
11:45 A.M.—240 c.c. sea-water poured off, 240 c.c. added.

At 11:45 A.M. 2 c.c. of eggs left. At 12:30 P.M. this supernatant sea-water gave a 6-second reaction at 1/100 dilution with *Arbacia* sperm. With *Echinarachnius* sperm the last positive reaction made with undiluted supernatant sea-water was at 9:50 P.M., July 12. 8:05 A.M., July 13, test was negative with *Echinarachnius* sperm.

The eggs used July 16 in the experiment cited above were farther washed as follows: 2:35, 3:06 and 5:15 P.M. by removing 90, 94 and 93 c.c. of sea-water, replacing in each case an amount equal to that withdrawn. The last positive effect with undiluted egg-water on *Echinarachnius* sperm was at 3:06 P.M. At 5:15 P.M. the last washing gave a positive reaction with *Arbacia* at 1/1,600 dilution.

In these washing experiments because eggs are lost, it might be argued that the hetero-agglutinin disappears because of the insufficient number of eggs left to charge the sea-water. But since the activity of the hetero-agglutinin in any case is inconstant, its loss in no wise paralleling that of the iso-agglutinin, this argument is scarcely tenable. Nothing is surer than the variation in hetero-agglutinin production by equal bulks of eggs from zero upward.

(*c*) *Precipitation of the Hetero-agglutinin by Echinarachnius Sperm.*—Thirdly, it can be shown that the agglutinin for *Echinarachnius* sperm present in sea-water above *Arbacia* eggs can be precipitated by *Echinarachnius* sperm leaving the *Arbacia* sperm agglutinin practically undiminished in strength. Thus, on July 15, I prepared three vials each with 1 c.c. of filtered *Arbacia* egg-water; to these vials, *A*, *B*, and *C* respectively were added 1, 2 and 4 drops of *Echinarachnius* sperm suspension. A fourth vial, *D* (control), contained 5 c.c. of *Arbacia* egg-water with no sperm. After five minutes the following results on *Echinarachnius* sperm were recorded: *A*, powerful agglutinant undiluted; negative at 1/8 dilution; *B*, positive; *C*, positive.

After ten minutes, *C* was negative; after fifteen *B* faintly positive; at twenty minutes all vials were negative.

Tested on *Arbacia* sperm, the vials *A*, *B* and *D* (control) gave a 6-second reaction at 1/400 dilution.

This experiment when repeated gave essentially the same results: the *Echinarachnius* sperm agglutinin may be completely removed, although this removal is not immediate, the rapidity with which the hetero-agglutinin is destroyed depending upon the density of the *Echinarachnius* sperm suspension used; the iso-agglutinin, on the other hand, remains undiminished in power.

(*d*) *Agglutinative Action of Arbacia Blood on Echinarachnius Sperm.*—Farther, it can be demonstrated that the perivisceral fluid of *Arbacia* has an agglutinative action on *Echinarachnius* sperm though lacking this action on *Arbacia* sperm. Thus on July 17, I carefully collected blood from four *Arbacia* which after clotting was filtered. Ten determinations on *Arbacia* sperm were negative in each case, the sperm showing, however, undiminished response to *Arbacia* egg-water tested after each trial with the blood. This perivisceral fluid agglutinated sperm from six *Echinarachnius* tested in turn.

Frequently, as in another observation on this same day, July 17, females were found whose blood was negative to both *Arbacia* and *Echinarachnius* sperm. Blood is positive with *Arbacia* sperm if the ovaries are ruptured when the animals are opened. Extreme care is necessary to avoid this.

(*e*) *Microscopic Appearance of Echinarachnius Sperm Agglutinated by Arbacia Egg-water.*—Finally, it may be noted that the microscopic appearance of *Echinarachnius* sperm agglutination by *Arbacia* egg-water is markedly different from agglutination by *Echinarachnius* egg-water. On this point I am borne out by four investigators who were good enough from time to time to observe the phenomena. In every instance they declared that they could note a difference in the character of the agglutination. When *Echinarachnius* egg-water is blown into a drop of *Echinarachnius* sperm suspension, the agglutination is sharply defined because after the initial activation the spermatozoa clump, leaving spaces between the clumps practictically free of sperm. After a time, depending upon the strength of the egg-water the clumps break up, the individual sperm being again homogeneously distributed throughout the field. With *Arbacia* egg-water the sperm of *Echinarachnius* behave differently. The agglutinated masses tend to string out with some free sperm among them. The agglutination is slower in beginning and with strong egg-water slow in breaking up, indeed may never break up, the agglutination being permanent. The whole picture suggests a coagulative effect. Agglutination of *Echinarachnius* sperm by *Arbacia* egg-water probably is much like that of *Nereis* sperm by *Arbacia;* this point I attempted to determine but could not succeed in getting *Nereis* sperm at the proper time.

The evidence here presented shows that the iso-agglutinin and hetero-agglutinin found in *Arbacia* egg-water are separate. What the source is, one can scarcely say, although it may well be the blood. Suspensions of eggs thoroughly washed free of perivisceral fluid would, therefore, fail to charge sea-water with the hetero-agglutinin, while the eggs would continue actively to produce the iso-agglutinin. Whatever, the source of the substance, the experiments cited above indicate that it is distinct from the iso-agglutinin and is of the nature of a toxic substance.

C. *Summary of Part III.*

Summarizing we find (1) that cross fertilization between *Echinarachnius* and *Arbacia* is possible but that the *Arbacia* egg

is greatly resistant to *Echinarachnius* sperm while the *Echinarachnius* egg is easily fertilized by *Arbacia* sperm. (2) *Echinarachnius* egg-water activates but does not agglutinate *Arbacia* sperm. *Arbacia* egg-water agglutinates *Echinarachnius* sperm. This is a hetero-agglutination by a substance in *Arbacia* egg-water separate from *Arbacia* fertilizin because it may be removed from the egg-water by dilution, by repeatedly washing the eggs and by precipitating it with *Echinarachnius* sperm. It is found in *Arbacia* blood. The microscopic appearance of this toxic hetero-agglutination of *Echinarachnius* sperm is different from that of the iso-agglutination.

IV. Discussion.

Every egg has a fertilizable period during which falls its optimum capacity for fertilization. Thus, *Asterias* eggs normally shed after complete maturation are capable of fertilization on immediate insemination but if procured by placing the ovaries in sea-water these eggs, in the germinal vesicle stage, fertilize only after the maturation process. Apparently, this is true of *Chætopterus* and *Cerebratulus* eggs which come into sea-water in the germinal vesicle stage: insemination is most effective after the germinal vesicle fades and the first maturation spindle is at the mesophase.[1] Immature eggs will not fertilize though sperm may bore into such eggs. Thus, in preparations of the normal fertilization of *Arbacia*, I find many ovocytes with one or more sperm in the cytoplasm, in some cases around the germinal vesicles. Such sperm undergo no change. Moreover, sperm may normally enter immature eggs according to Hempelmann, Shearer and von Hofsten. Sperm entry, therefore, is no criterion of fertilization. Moreover, the fertilizable period of an egg gradually passes over as can be shown by the process of staling, allowing the eggs to stand in normal sea-water. This may take place as in *Platynereis* in an almost incredibly short time; in *Echinarachnius* the time before capacity for fertilization is lost is much longer though not so long as in *Arbacia*. Sperm may

[1] Wilson has found that in *Cerebratulus* after the germinal vesicle has broken down, merogony is possible; enucleated fragments after insemination are not capable of refertilization. (Cf. also Delage.)

enter these stale eggs likewise; for example, in *Platynereis* (Just, '15*b*) sperm not only enter but come into apposition with the female nucleus without inducing development though maturation has been complete. Through the work of Lillie on *Arbacia* these phenomena may be interpreted: immature eggs, stale eggs and fertilized eggs produce no fertilizin; they are incapable of fertilization. One cannot help but postulate a causal connection. Much the same interpretation is possible in *Nereis* and *Platynereis*. In *Echinarachnius* the process is in every way similar to that in *Arbacia*. Whatever prejudice one may have to the fertilizin theory of fertilization because of the immunological terminology, the proved facts one must admit. At the very least, fertilizin is an index to fertilization capacity.

Again, eggs show a period during which they are most susceptible to agents that initiate development. This has been most clearly shown for the starfish by Mathews and by R. S. Lillie. It is generally known that the eggs of some females respond more readily to agents that initiate development than others. In the *Nereis* (Just, '15*a*) one may observe a very striking behavior of the egg to warmed sea-water. From a female quickly dried, dry eggs are taken and divided into two lots. One lot is placed in a small quantity of sea-water at 33° C. and the other into ordinary sea-water. Those eggs in warm sea-water will form jelly, maturate and cleave, the resulting embryos closely simulating the normal. If the eggs in ordinary sea-water be transferred to warmed sea-water *they do not even form jelly*. Nothing is more striking than the comparison between two lots of *Nereis* eggs that have been subjected to warming with and without previous exposure to ordinary sea-water: the dry eggs are in a thick mat of jelly, the washed eggs closely crowded together without any jelly formed. In *Nereis*, then, the egg must have its full content of fertilizin in order to respond to warming as a means of artificial parthenogenesis: it loses its power for initiation of development by artificial means before it loses its fertilizing power. Muscle, nerve and gland respond to artificial stimulation, but the normal mode is most effective. So the sperm is the most efficient activator for the egg. In *Nereis* the best time for artificial parthenogenesis is the time of greatest fertilizin content.

Farthermore, the studies of Lillie have shown that like the fertilized egg, the egg induced to development artificially no longer produces fertilizin. Moore's ('16, '17) studies confirm and extend those of Lillie.

The presence of fertilizin in *Echinarachnius*, *Arbacia* and *Nereis* is indicated by its power to agglutinate specific sperm. But this very agglutination of the sperm is held as an argument against the fertilizin theory because it is held to "inhibit the fertilizing effect of the spermatozoa instead of enhancing it since the cluster formation prevents the sperm from reaching the egg. Even from a teleological viewpoint it is difficult to understand why a substance which only prevents the fertilizing action of the sperm should be a necessary link in such action" (Loeb, '14). First, however, it should be recalled that this agglutination is a reversible process where specific sperm are employed, the clumping lasting from ten to twenty seconds for average egg-water dilution. Second, while Lillie found that agglutination reduces the per cent. of fertilization, Fuchs observed the contrary to be the case. Finally, we should note these agglutinations are made in the laboratory with fairly thick sperm suspension and that not only the motility of the sperm but the density of the suspension are functions in the agglutination reaction. Agglutination would scarcely take place in the sea where the conditions are not those in a drop of water on a slide. As a matter of fact agglutination rarely takes place during insemination in a finger-bowl because we very carefully use thin sperm suspensions to avoid polyspermy. The sperm are thus too widely separated to clump; instead, they stick to the eggs. I, therefore, heartly agree with Loeb when he states that he is "inclined to believe that the cluster formation or agglutination of sperm does not occur when fertilization takes place under natural conditions." But this in no wise invalidates the claims of the fertilizin theory.

Concerning the sperm agglutination by egg-water of another form, hetero-agglutination, it seems to me that it would be necessary to know the *nature* of the agglutination; this demands the microscopic method of studying the agglutination. Thus, *Arbacia* egg-water agglutinates *Nereis* sperm, but this is a toxic

agglutination produced by a substance distinct from the iso-agglutinin; it is found in *Arbacia* blood. Likewise the agglutinin in *Arbacia* egg-water for *Echinarachnius* sperm is separate from the iso-agglutinin. These points could be better determined by the microscopic method, by examining the agglutination after diluting the egg-water, attenuating the agglutinin by washing the eggs, etc. The macroscopic method of studying the agglutination would fail. It may be, farthermore, that *Arbacia* blood is generally toxic. Thus, I have found that it will induce jelly-formation, maturation, and if allowed to act too long cytolysis in the *Nereis* egg. We may readily understand that when cross fertilization is possible between forms in one direction only the fact that an agglutination takes place in the opposite direction does not invalidate the fertilizin theory of fertilization if the agglutination is a toxic hetero-agglutination.

Finally, the activation of sperm by egg-water may be of great importance in fertilization as Loeb ('15) points out. Thus, *Echinarachnius* egg-water activates *Arbacia* sperm which are capable of fertilizing *Echinarachnius* eggs. In *Asterias* if at the moment of insemination by immobile sperm more sperm be added they are greatly activated. In *Nereis* agglutination is only possible with very active sperm; such sperm spontaneously form aggregations when mixed with sea-water alone. Sperm that do not form these spontaneous aggregations will not agglutinate. Also in both *Arbacia* and *Echinarachnius* the first stage in the agglutination reaction is activation. Probably in those *Echinarachnius* eggs normally resistant to fertilization there exists a membrane condition that masks the presence of fertilizin. In addition, therefore, to the farther study of activation we must approach the investigation of just such conditions. By these analyses the fertilizin theory may prove of wider application.

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THE FERTILIZATION REACTION IN ECHINARACHNIUS PARMA.

III. The Nature of the Activation of the Egg by Butyric Acid.

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I. Introduction.

The egg of *Echinarachnius* as shown elsewhere (Just, '19*a*) following engulfment of one sperm immediately becomes immune to farther sperm entry. This change takes place in the cortex *before* the membrane lifts off and probably represents the neutralization of the fertilizin by the anti-fertilizin present in the egg. This is the mechanism for the prevention of polyspermy, and not the immediately succeeding membrane lifting. Loeb, however, found that butyric acid, though it be capable of calling out "fertilization membranes" in one hundred per cent. of the eggs of the sea-urchin, is none the less incomplete in its action, for if by shaking he removes these "fertilization membranes" sperm penetrate the eggs, membranes form anew, and development proceeds like the normal. This finding certainly does not support his view that fertilization is a kind of artificial parthenogenesis for after fertilization eggs or fragments thereof do not

"refertilize" (see older work of Driesch and the important work of Wilson on *Cerebratulus*). The present paper reports results of an attempt to determine whether or not in *Echinarachnius* eggs activation by butyric acid is complete in the sense that it is complete when membranes form after the initial stage in the fertilization process. If we assume that activation is complete in eggs that have formed membranes after butyric acid then hypertonic sea-water exposure should give the best development with these eggs. Any other assumption is absurd unless the use of butyric acid is unnecessary, when used with hypertonic sea-water, for the best type of development. Again, on the assumption that with membrane formation following butyric acid the activation is complete, as it is in fertilization, the egg should react on insemination as do fertilized eggs or fragments thereof. The observations show that after butyric acid treatment under optimum conditions eggs form membranes (Part II.); and that in such eggs activation is complete since with hypertonic treatment they give rise to cleavages, gastrulæ and plutei of a high degree of normality, and since they fail to develop after insemination (Part III.).

II. Activation of Echinarachnius Eggs by Butyric Acid.

Eggs of *Echinarachnius* if properly exposed to butyric acid in sea-water are activated and so form membranes that are indistinguishable from those formed following insemination.

A. *Optimum Exposure to Butyric Acid.*

Without the experiments being cited it may be said that by using various mixtures of butyric acid in sea-water the best mixture was found to be 2 c.c. of *n*/10 butyric acid plus 50 c.c. of sea-water. The optimum exposure for this mixture discovered by removing samples of eggs from the butyric mixture at five second intervals, is thirty-five seconds. In some cases eggs were exposed for sixteen minutes.

The following experiment of July 8 is cited as typical: 9:50 A.M., 15 dishes each with 250 c.c. of sea-water. 10:18 A.M., 2 c.c. *n*/10 butyric acid plus 50 c.c. of sea-water thoroughly

mixed. 10:20 A.M., shed eggs from one very fine female exposed to butyric mixture, samples being removed at intervals to dishes of 250 c.c. of normal sea-water. Table I. gives the data.

TABLE I.

No.	Length of Exposure.	Per Cent. of "Full Membranes."
1	20 seconds	14
2	25 "	72
3	30 "	85
4	35 "	93
5	40 "	87
6	45 "	83
7	2 minutes	0
8	5 "	0
9	6 "	0
10	7 "	0
11	8 "	0
12	9 "	0
13	10 "	0
14	11 "	0
15	12 "	0

Eggs of nos. 7–15 showed a tendency to go bad; this increased with the length of exposure, for no. 15 eggs were very bad.

The results are essentially the same in the experiments of July 11 and July 26 as shown in Table II.

TABLE II.

No.	Length of Exposure.	Per Cent. of "Full Membranes."	
		July 11.	July 26.
1	20 seconds.	3	18
2	25 "	39	36
3	30 "	50	76
4	40 "	96	91
5	45 "	67	83
6	50 "	49	—[1]
7	55 "	12	—[1]
8	60 "	1	1
9	65 "	0	—[1]

[1] No exposure for this length of time.

During August 4, seven lots of eggs from different females were exposed to 2 c.c. *n*/10 butyric acid plus 50 c.c. sea-water for 20, 25, 30, 35, 40, 60, 90 and 120 seconds. The 35-second exposure in each case gave 91, 90, 98, 93, 99, 97 and 97 per cent. full membranes. Observations of August 6 were about the same.

These citations are sufficient, I think, to show that there is a very definite optimum exposure between rather narrow limits for the activation of *Echinarachnius* eggs by the mixture of butyric acid and sea-water employed. But if the eggs are exposed to *higher* concentrations of butryic acid even for a shorter time they do not form full rounded membranes that stand off from the cortex but thick membranes adhering closely to the cytoplasm. Thus, on July 6, eggs exposed to 3 c.c. of *n*/10 butyric acid plus 50 c.c. of sea-water and those exposed to 3.5 c.c. of the acid plus 50 c.c. of sea-water for 20 seconds each gave one hundred per cent. of these thick contracted membranes. These membranes are entirely different from the so-called "fertilization membranes" got with lower concentrations of butyric used. Apparently, Loeb obtained the same results with *Arbacia* eggs. Speaking of the effect of butyric on this egg he says: "When transferred to sea-water, they did not form a conspicuous fertilization membrane as did the eggs of *S. purpuratus* under the same circumstances, but only a fine gelatinous layer which was not easily visible" (page 71).

On shaking some time after removal from butryic sea-water full membranes partially collapse; contracted membranes on shaking break and the eggs give off buds. These buds show extreme variations in size. Butyric treatment alone (at least for the longest exposure tried, sixteen minutes) never gives cleavage. The eggs never go beyond the monaster stage.

B. *Condition of the Eggs as a Factor in the Response to Activation by Butyric Acid.*

Without exception my best results with *Echinarachnius* eggs have been obtained with dry shed eggs—*i. e.*, eggs deposited by the animals in clean dry watch glasses. It is not always practicable to wait until the animals shed; I, therefore, proceed as follows: The animals are washed in sea-water then in running tap-water, shaken dry and placed aboral side down after a slight cut around the peristome which usually suffices to induce shedding;[1] such eggs are scarcely inferior to normally shed eggs. In

[1] Great care was exercised to keep everything sterile. On only one occasion was an egg found in the two cell stage in the control—not more certainly than one in three or four hundred eggs, but the experiment was discarded.

the experiments cited above the butyric mixture previously prepared was added directly to the Syracuse glass containing the eggs. I was content to work with relatively a small number of eggs always from but one female.[1] The amount of butyric mixture used, therefore, could be likewise small (in my experiments never more than 12.5 c.c.) so that the eggs could be thoroughly stirred up and still settle quickly with the result that when at most the 1 to 1.5 c.c. was removed the amount of acid carried over was negligible.[2]

1. *Effect of Washing.*—Dry eggs may be washed quickly once or twice in a small amount of sea-water without affecting the butyric treatment. Additional washings will cut down the per cent. of butyric membranes and washings extending over one to two hours will result in the failure of butyric acid to form membranes. Eggs similarly treated may be still capable of fertilization. Eggs incapable of fertilization never respond to butyric acid treatment.

An experiment of July 26 shows the difference between dry and washed eggs:

8:55 A.M. A fine lot of eggs divided among three dishes—*A*, *B* and *C*. Lot *A* dry; Lot *B* washed three times in fifty minutes; to lot *C* two drops of body fluid added.

10.00 A.M. Lot *B* gave the following results after exposure to butyric sea-water:

Exposure in seconds	20	25	30	35	40	60
Per cent. of membranes	6	19	38	27	14	−1
Lot *A* (unwashed) per cent. of membranes	18	36	76	91	83	1

On August 4 similar results were obtained. After four washings in thirty minutes the per cent. of membranes formed was reduced one half after optimum exposure as compared with the unwashed.

[1] Indeed, in my judgment the practise of using large quantities of eggs from a number of females is a most pernicious one, introducing as it does more unknown factors. Nothing is so variable as the echinid egg, as Tennent, Medes and Goldfarb have shown—the work of the last-named being particularly concerned with our problem; in a lot of eggs from several females one must get a very heterogeneous population—especially, as is usually the case, if the worker obtains his eggs by mincing the ovaries in sea-water.

[2] Transfers were sometimes made to 4,000 c.c. of normal sea-water; the results obtained were no better than those to 250 c.c. of sea-water.

Against these observations the objection might be raised that the failure of washed eggs to form membranes in greater number is simply due to the low concentration of butyric acid in sea-water since from the washed eggs all the water cannot be removed. Against this we have (1) the fact that dry eggs quickly washed once or twice may give one hundred per cent. membranes. If, by chance, body fluid was present with shed eggs they were always washed. (2) After washing all but a negligible amount of water can be removed. (3) Diminution in concentration of acid does not explain absolute loss by the egg of the power to respond to butyric acid which may take place in an hour. (4) Finally, washed eggs subjected to butyric acid in higher concentration (*e. g.*, 3 c.c. *n*/10 butyric plus 50 c.c. of sea-water) if they form membranes at all form the thick closely adherent membranes which result from exposure to too great concentration. Washing alone is responsible for the failure of the eggs to form membranes; repeated washings in 10 to 15 c.c. of sea-water for one to two hours or a single washing in 250 c.c.[1] will render eggs incapable of activation by butyric.[2]

2. *Effect of Body Fluid.*—My 1914 experiments on the initiation of development of *Nereis* eggs through increased temperature proved that serum as definitely inhibits initiation of development, as it does fertilization (Just, '15). I was, therefore, led to study the effects of *Echinarachnius* body fluid on butyric acid activation of *Echinarachnius* eggs. Eggs mixed with body fluid form few if any full membranes; they usually form the thick closely adherent type of membrane.

(*a*) Thus, the eggs of Lot *C* of July 26 mentioned on page 43 (eggs plus two drops of body juice) gave the following:

Exposure in seconds	20	25	30	35	40	60
Per cent. of membranes	0	3	14	16	11	0

(*b*) July 15, 10:30 A.M. Eggs plus body fluid (2 drops each) in butyric acid sea-water for 20, 25, 30, 35 and 40 seconds.

10:50 A.M. *Not one full membrane;* some eggs have the thick contracted membranes.

[1] I did not wash in the larger quantities often, for with them the difficulty of recovering the small number of eggs increases; moreover, concentrating the eggs takes time—thus introducing another factor.

[2] Washing may not have the same effect on *Arbacia* eggs which are much more resistant. (See Just, '19*b*.)

(*c*) July 8. Eggs shaken from ovaries into butyric mixture gave the following results:

Exposure in seconds	20	25	30	35	40	45	50	55
Per cent. of membranes	33	38	34	48	31	20	17	0

All contracted membranes.

(*d*) Experiments of August 4 and 6. Serum eggs form no membranes after butyric acid exposure.

In (*d*) the cortical changes that are responsible for membrane formation were probably complete (see beyond). The actual lifting off of the membrane is not a *sine qua non* of activation. (See Just, '19*b*. Loeb, '15, discusses this point apropos certain observations of Brachet.)

Heilbrunn found that in *Arbacia* eggs membrane elevation by acid is inhibited by *Arbacia* blood.

C. *Summary of Part II.*

Summarizing we find that butyric acid in sea-water activates *Echinarachnius* eggs if they are in best physiological condition; slight washing will not impair their capacity to respond to the acid but long washing will. The body fluid inhibits membrane elevation.

III. Analysis of Butyric Acid Activation.

If membrane elevation is a sign of complete activation those eggs exposed for optimum length of time to sea-water with butyric acid in optimum concentration should respond best to subsequent hypertonic treatment. Moreover, activation by butyric if complete as in fertilization should inhibit fertilization. The present section deals with the analysis of butyric acid activation and contains the evidence that shows that such activation is complete.

A. *Effect of Hypertonic Sea-water on Butyric Treated Eggs.*

Butyric acid acting along on *Echinarachnius* eggs (at least for exposures employed which were up to 16 minutes) never induces cleavage. But after butyric activation hypertonic sea-water causes cleavage—62 per cent. in one case—which is scarcely to be distinguished from the normal; such eggs form gastrulae and

develop into plutei that swim to the top of the dishes. Mixtures in the proportions 5, 6, 7 and 8 c.c. of 2.5 M NaCl plus 50 c.c. of sea-water in each case were employed. The best results were obtained with the mixture of salt and sea-water in the proportions of 5 cc.. of 2.5 M. NaCl plus 50 c.c. of sea-water.[1] The procedure was as follows: Eggs exposed to butyric acid sea-water were removed at intervals of 5 seconds to 250 c.c. of sea-water. Following about 20 minutes in sea-water eggs were carried over to dishes of hypertonic sea-water at 15, 20, 25, etc., minutes. Twenty-five minutes treatment is best. *Eggs previously exposed to butyric acid for 35 seconds (optimum exposure for membranes) gave the highest per cent. of cleavage and the best type of development.*

It would be tedious to cite individual experiments on this point. The reader would scarcely be interested in experimental details on the rather threadbare subject of the effect of hypertonic sea-water following butyric activation. It is, of course, generally understood that hypertonic sea-water is most successful subsequent to butyric acid treatment where membranes have been formed. There would certainly be no reason to use butyric acid if this were not true. Notwithstanding all this, I wish to emphasize that those eggs with the highest per cent. membranes (optimum exposure) yield best to hypertonic treatment. Eggs under- or over-exposed to butyric acid do not give the same type of development.[2]

B. *Effect of Insemination Following Butyric Acid Activation.*

After removal to normal sea-water following optimum exposure to butyric acid sea-water *Echinarachnius* eggs form 100 per cent.

[1] The 2.5 M. NaCl solution was always freshly made. No solution was ever older than six hours when added to the sea-water.

[2] The successfully treated eggs are certainly very beautiful; as far as the gastrula stage in most cases they appear to be perfectly normal. The plutei develop more slowly than those from fertilized eggs and are not so hardy. Possibly, with even nicer treatment, the development could be improved.

Cytological study of eggs induced to develop with artificial means has been made by Wilson, Hindle and Herlant. In the living *Echinarachnius* egg we observe that while in sea-water following butyric acid treatment a monaster forms beyond which stage the egg does not develop. Having been properly exposed to hypertonic sea-water the egg forms in sea-water a complete astral system. Overexposure to hypertonic sea-water results in the formation of innumerable asters when the egg is removed to sea-water: such eggs fail to cleave or form very irregular cleavages.

membranes in highly successful cases. Such eggs mixed with sperm do not develop though the membranes be previously removed as soon as formed. Thus, activation with butyric acid is complete; eggs with membranes have undergone an irreversible change.

The Experiments.—The method followed in these experiments is simple. Eggs are exposed to $n/10$ butyric acid in sea-water (2 c.c. of acid plus 50 c.c. sea-water) and removed to 250 c.c. of normal sea-water at five second intervals; these dishes comprise Series *A*. From the dishes of series *A* eggs are removed, shaken as soon as membranes are formed, and inseminated with fresh sperm suspension; thus, series *B* is established. Samples of eggs from each of the *A* dishes inseminated without shaking off the membranes make up series *C*. The membranes formed in series *A* and later the cleavages in *B* and *C* are recorded; *B* and *C* dishes are then gently washed in fresh sea-water and set aside for study of the development during the next twenty-four hours.

The experiment of July 9 is typical: 10.00 A.M. 6 dishes each with 250 c.c. of sea-water to which eggs are removed after exposure to butyric sea-water after 30, 35, 40, 60, 120 and 180 seconds (series *A1* to *A6*). Samples from *A1* to *A6* shaken and inseminated (series *B*, 1 to 6). Series *C* samples from *A1* to *A6* inseminated without shaking. Table III. gives the results:

TABLE III.

No.	Exposure in Seconds.	Per Cent. of Membranes in *A*.	Per Cent. of Cleavages,		Per Cent. of Swimmers.	
			In *B*.	In *C*.	In *B*.	In *C*.
1	30	70	27	28	7	6
2	35	76	30	27	6	3
3	40	74	29	23	0	1
4	60	5	13	3	2	4
5	120	0	30	43	44	41
6	180	0	48	44	41	39

B4 to B6 and C4 to C6: cleavages very irregular; "swimmers" extremely abnormal—exogastrulæ, microgastrulæ, blastulæ with dense opaque interiors evidently due to abortive gastrulation.

I have given this particular experiment because not a very high per cent. of the eggs formed membranes. This ought, therefore to be crucial, for since under-exposed eggs (*i. e.*, those which are

do not form membranes after short exposures) may fertilize perfectly we may assume that these particular eggs were on the border line of proper exposure. Insemination, however, caused no membranes (cf. Herbst) in the over-exposed eggs, and this is always the case, though some of the eggs cleave.

Membranes once formed may be removed, but after insemination these eggs develop no farther than uninseminated butyric treated eggs. Study of the figures given in Table III. shows first, that the cleavages of unshaken inseminated eggs (with membranes) and of shaken inseminated eggs (without membranes) are approximately equal; and second, that the per cent. of membranes in *A* plus the per cent. of cleavages in *B* or *C* is very close to a hundred. This must mean that eggs with membranes formed with butyric acid are not fertilizable. In a given lot of eggs exposed for the optimum time to butyric some are not completely activated and have not formed membranes. These are the eggs that cleave following insemination; the removal of the membranes does not affect the per cent, of cleavages thus obtained.

The experiment of July 8 part of which has already been given (p. 40) is clearly decisive. Following exposure to butyric sea-water Series *A* eggs were shaken and inseminated (series *B*) with the results found in Table IV.

TABLE IV.

No.	Exposure.	Per Cent. of Membranes (Series A).	Per Cent. of Cleavage After Insemination (Series B).
1	20 seconds.	14	50
2	25 "	72	26
3	30 "	85	15
4	35 "	93	6
5	40 "	87	16
6	45 "	83	19
7	2 minutes	0	41
8	5 "	0	43
9	6 "	0	—
10	7 "	0	—
11	8 "	0	—
12	9 "	0	—
13	10 "	0	—
14	11 "	0	—
15	12 "	0	—

B7 to B15: cleavages very abnormal; frequently incomplete many spindles present.

July 26, August 4 and August 6. Eggs after 35 seconds exposure showed 100 per cent. membranes but failed to develop following insemination whether freed of membranes or not.

Early in the season eggs were found that failed to form a single membrane subsequent to optimum butyric treatment. Such eggs on insemination never developed. Eggs inhibited by the body fluid may not form membranes; if they be inseminated they do not form membranes or otherwise develop. Such eggs have undergone cortical changes but membrane formation is suppressed. In serum eggs the suppression usually is not complete: the membrane thickens and remains stuck to the egg.

Moore working with *Arbacia* found that those eggs that form butyric membranes do not develop after insemination though the sperm may penetrate. Whether under similar conditions sperm enter the eggs of *Echinarachnius* I am unable to say. Mere penetration, however, in no wise affects our position. Development does not depend on sperm penetration simply; development begins with certain cortical changes which having been initiated by agents other than the sperm inhibit action by superimposed sperm.

C. *Summary of Part III.*

We may summarize the results of this section as follows: Butyric acid eggs with membranes respond best to hypertonic sea-water giving development that closely simulates the normal. Eggs with butyric membranes cannot be fertilized though the membranes be removed. These facts indicate that cortical activation is complete.

IV. Discussion.

1. Workers generally appreciate how capricious is the response of eggs to agents that initiate development. Often the procedure of a given experiment may be repeated with altogether different results. Hence we conclude that the varying "physiological condition" of the eggs is responsible for our results. Study of fertilization in *Echinarachnius* (Just, '19*b*) shows that this physiological condition depends on the presence of fertilizin: eggs that have a high fertilizin index show a high per cent. of

development; eggs washed free of fertilizin lose their fertilizing power; immature eggs and fertilized eggs show no fertilizin reaction and are incapable of fertilization (and re-fertilization).

Now, development of sea-urchin ova and of marine ova generally by chemical and physical agents is of course purely artificial; it is at least debatable that these agents are as efficient for initiating development as specific sperm. In *Nereis* and in *Echinarachnius* the volume of evidence is incontrovertible: only those eggs in best condition (as shown by their fertilizin tests) respond to artificial agents that initiate development. If eggs from a given lot fail of fertilization the remainder of that lot will not respond to artificial agents. Indeed, in both *Nereis* and *Echinarachnius* the egg through washing—and loss of fertilizin—loses its power to respond to warming (*Nereis*) or to butyric acid (*Echinarachnius*) before it loses its power to respond to sperm. Eggs with full fertilizin content most nearly ensure success with artificial agents.

Blood inhibits fertilization—a truism to the embryologist; where marine ova are normally shed and insemination takes place in the sea, it is generally found best to wash the eggs before insemination. Normally shed eggs always give a high per cent. of development; eggs cut or washed from the ovaries are not so good. Thus, in *Podarke* and in *Cumingia*, for example, eggs develop in greatest number if laid. The blood blocks fertilization "by occupying the ovophile group of the fertilizin, thus preventing action of the latter upon the egg by union with egg receptors" (Lillie). It probably acts in the same way in artificial initiation of development.

In *Nereis* and in *Echinarachnius* blood inhibits both fertilization and artificial initiation of development. In *Arbacia* Lillie found that blood inhibits fertilization while Heilbrunn observed that it also inhibits membrane formation. The worker, therefore, who studies "artificial parthenogenesis" is between Scylla and Charybdis—either he will not wash the eggs and thus have blood inhibitor present, or he will wash too much and so bring about too great secretion of fertilizin. This may seem fanciful to some but experience with *Nereis*, *Platynereis* and *Echinarachnius* teaches that the egg is anything but an inert cell; for suc-

cessful treatment with artificial agents it must be handled with extreme care to insure uniform results.

2. *Echinarachnius* eggs that form membranes through butyric acid treatment are completely activated since such eggs respond to subsequent hypertonic sea-water treatment with development that closely simulates the normal, and since eggs under- or over-exposed to butyric acid and which do not form membranes never form membranes following hypertonic sea-water treatment nor do they develop at all normally. Activation, again, must be regarded as complete since butyric acid eggs with membranes fail to develop after insemination even though the membranes be removed as soon as formed; in this they resemble fertilized eggs or fragments thereof that fail to re-fertilize. Finally, these butyric acid treated eggs with membranes fail to give the fertilizin test as rapidly as do fertilized eggs. We must, therefore, conclude that activation is as complete in eggs with butyric membranes as in eggs with fertilization membranes.

It is interesting to note that Loeb found that his sea-urchin's eggs after butyric acid membrane formation were capable of fertilization provided he had removed the membranes by shaking. Lillie, however, found that *Arbacia* eggs after membrane formation by butyric acid cease to produce fertilizin as do fertilized eggs. Moore confirmed Lillie's butyric abservation on *Arbacia* and also discovered that eggs with butyric membranes are incapable of fertilization. To Loeb, complete membrane formation by butyric is an incomplete activation; to the workers with *Arbacia* and *Echinarachnius* eggs membrane formation by butyric acid is a complete activation. In initiation of development, therefore, the egg gives a similar response both to the artificial agent and to the spermatozoa. This, however, does not entail subscription to the famous "lysin theory."

It is now some twenty years since the work on artificial parthenogenesis began. During this time eggs have been subjected to every kind of agent imaginable, a vast amount of literature has been produced, and many guesses have been made on the basis of this work as to the nature of the fertilization reaction. This work on artificial parthenogenesis constitutes one of the important chapters in the history of modern biology and no

mean part among the many brilliant achievements of Loeb which aside from their inherent value have another claim to distinction—their stimulating effect on the new experimental zoölogy. But *artificial parthenogenesis is not fertilization*. And it is indeed unfortunate that the term artificial parthenogenesis ever had that connotation. Thus we read of "fertilization membranes" by this or that chemical, "fertilizing agents," "chemical fertilization," etc., when we know that there are eggs that will respond to very slight changes in the environment. The eggs of *Amphitrite*, *Asterias*, *Nereis*, etc., for example, will respond simply to agitation. Artificial parthenogenesis *per se* is too significant to masquerade as fertilization. The processes initiated in artificial parthenogenesis and fertilization probably are the same. But to argue that the sperm carries a lysin because a host of agents activate the egg is bad logic. It is far simpler to postulate that the egg contains the necessary mechanism for development. Such a postulate embraces not only artificial parthenogenesis and fertilization, but also the widely occurring and *real* parthenogenesis. Artificial parthenogenesis teaches us that the egg is a highly irritable cell that will respond to various agents; it has placed us but little nearer a solution of the fertilization problem than we were twenty years ago. One might as easily deduce from the contraction obtained on a curarized muscle with any one of a number of stimuli the nature of the nerve impulse as to formulate a theory that since CO_2, or any other of a number of agents induces development fertilization is due to a lysin carried by the sperm.

Nor yet have purely morphological hypotheses proved satisfactory. The Boveri hypothesis as its predecessors is no longer considered adequate; and such deductions as those of Meves lead only to absurdities.

Lillie's assumption that the egg in its fertilizable condition contains a substance (fertilizin) which needs merely to be activated is logical and is based on observation and experiments which themselves, theory aside, excite admiration. Future work will determine how much this theory has helped toward a solution of the fertilization problem.

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THE EFFECTS OF A DIET OF POLISHED AND OF UNPOLISHED RICE UPON THE METABOLIC ACTIVITY OF PARAMECIUM.[1]

MARY DRUSILLA FLATHER.

The purpose of the following experiments was to discover the effects of the presence and absence of the vitamin contained in rice upon the metabolic activity of *Paramecium*. It was successfully proved by Funk (2), that the vitamin which prevents the deficiency disease known as beri-beri in man is removed from the rice by the process of polishing. Since the lack of this vitamin in the diet of human beings produces a seriously diseased condition it seemed a promising subject for research to determine whether or not the effects could be so far-reaching as to alter the metabolism of a one-celled organism, *Paramecium*. Calkins (1) had previously shown that pancreatic vitamin, a specific in the case of marasmus, had no influence whatever upon the metabolic activities of *Paramecium*. Calkins used the division rate as an index to the vitality of the organism, believing that certain substances in the culture medium may increase the rate of division and others decrease it. This criterion is the one used in the following investigation.

The experiments were performed upon individuals from five pure lines, all from wild cultures started in the laboratory at the same time and kept in spring water and hay infusion for one month. The pure lines were started on December 4, 1917. Each individual was isolated in three drops of distilled water and one drop of 1 per cent. malted milk solution, renewed every other day. The malted milk was prepared by pouring 50 c.c. of boiling distilled water over .5 gram of Horlick's malted milk. After several divisions had taken place each pure line was transferred from the depression slide to a large stock dish containing 200 c.c. of distilled water and 5 c.c. of malted milk solution. Once a week one half of the stock solution was removed and an

[1] From the Biological Laboratory of Bryn Mawr College.

equal amount of fresh water and 5 c.c. of fresh malted milk were added.

In order to eliminate as far as possible the influence of unknown variables every effort was made to prevent contamination of the culture media; graded pipettes were used in measuring the media and the slides containing the individuals were kept in a moist chamber at constant temperature, and removed for observation at approximately the same hour every day. Individuals for the special problem were isolated on March 9, 1918, three being taken from each pure line. Of these three, one was isolated on a depression slide in two drops of distilled water and two drops of 1 per cent. rice water made from polished rice, and a third in two drops of distilled water and two drops of 1 per cent. rice water made from unpolished rice. The rice water was prepared by boiling .5 gram of finely ground rice in distilled water and making up the solution to 1 per cent. after evaporation. The number of divisions was noted at the end of every twenty-four hours, and one individual from each group was isolated on a clean slide in the same medium as before. While the bacterial content is a questionable factor in the experiments, it is controlled to a large extent by the sterilization of slides and pipettes, by the boiling of all media and by the daily isolation of the individuals in fresh media.

Some trial experiments were made for perfection of technique, and then, using eleven days as a fair unit of time, final averages were made of the rates of division in each group. The individuals in malted milk showed the normal rate of division which was slightly higher than that in hay infusion. Peebles (8) has shown that if rightly used a weak solution of malted milk is a most satisfactory culture medium. The metabolic activities of the individuals in polished and in unpolished rice exhibited the influence, not only of the presence and absence of the vitamin, but also of the change of environmental conditions as represented by a new culture medium. Results are given in the accompanying tables.

The number of divisions during the first twenty-four hours of experimentation are not given in the tables, because some time must be allowed for adaptation to new conditions.

TABLE I.

THE DAILY RATE OF DIVISION.

Line.	Medium.	2d Day.	3d Day.	4th Day.	5th Day.	6th Day.	7th Day.	8th Day.	9th Day.	10th Day.	11th Day.
1	Polished rice	0	0	1	0	1	0	1	0	0	0
1	Unpolished rice	1	0	0	1	1	0	1	0	0	1
1	Malted milk	1	1	1	1	1	1	1	1	0	1
2	Polished rice	0	1	0	0	1	0	1	0	1	0
2	Unpolished rice	0	1	1	1	1	1	1	1	0	1
2	Malted milk	1	1	1	1	1	1	1	1	1	1
3	Polished rice	0	1	0	1	0	0	1	0	1	0
3	Unpolished rice	1	0	0	0	2	0	1	1	0	1
3	Malted milk	0	1	0	1	0	2	1	1	1	1
4	Polished rice	0	1	0	1	0	0	1	0	0	0
4	Unpolished rice	0	1	1	0	1	0	0	1	0	0
4	Malted milk	0	1	0	1	1	1	1	1	0	1
5	Polished rice	1	0	0	1	0	0	1	0	0	0
5	Unpolished rice	1	0	1	1	0	0	1	0	1	1
5	Malted milk	1	1	0	1	1	0	0	1	1	2

TABLE II.

TOTAL OF DIVISIONS IN EACH GROUP AND AVERAGES.

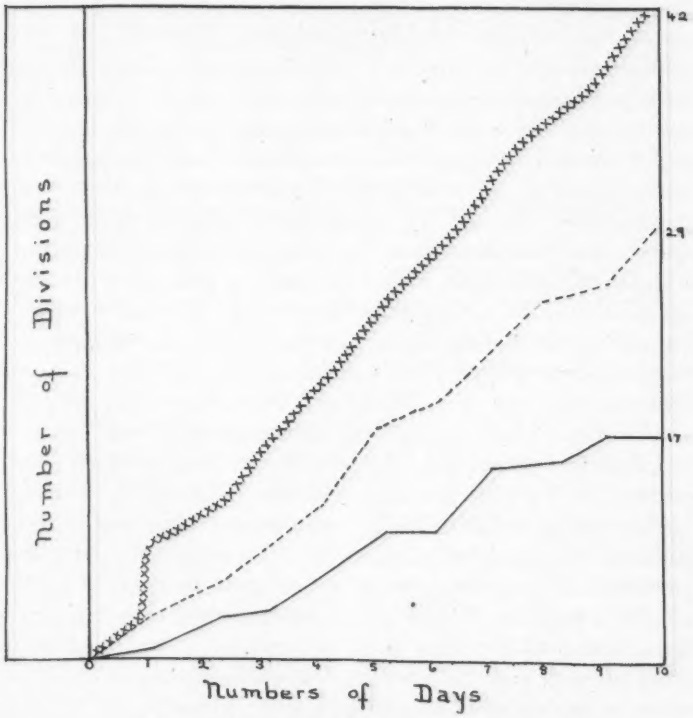
Medium.	Total of Divisions.	10-day Average.	Daily Average.
Polished rice	17	3.4	0.34
Unpolished rice	29	5.8	0.58
Malted milk	42	8.4	0.84

The graph shows that the division rate with polished rice is broken and irregular, while that of the unpolished rice is fairly regular and that of the malted milk very regular in daily increase in the number of divisions. From these results it is evident that unpolished rice is much less favorable than malted milk as a culture medium for *Paramecium*, and that polished rice is quite inadequate for maintaining the vitality of the organisms. It seems probable that the essential factor lacking in the diet of polished rice is the vitamin removed in the process of polishing.

According to McCollum and Davis (4) polished rice is deficient in fat-soluble A, and water-soluble B, the accessory food substances necessary for normal growth and maintenance of life. The fats of rice contain very little if any fat-soluble A, while all the water-soluble B is carried in rice polishings. In using

polished rice as a diet for young rats McCollum and Davis found it necessary to supplement the diet with water-soluble and fat-soluble vitamines. They found that 2 per cent. skim milk powder in combination with the rice supplied nearly enough water-soluble B for normal growth. In the experiments with

TABLE III.



GRAPH SHOWING DAILY TOTAL FOR EACH GROUP.

Crosses indicate divisions of individuals in malted milk. The dotted line indicates divisions of individuals in unpolished rice. The continuous line indicates divisions of individuals in polished rice.

Paramecium the addition of one drop of 1 per cent. malted milk solution to one drop of 1 per cent. polished rice water and two drops of distilled water restored the division rate to the normal

rate shown in malted milk alone. The efficacy of malted milk is due to the presence in it of both water-soluble B and fat soluble A. Unpolished rice contains both but not enough fat-soluble A for maintenance (6).

Since orange juice is so potent a specific in the treatment of deficiency diseases in man, a second set of experiments was undertaken to see if when used with polished rice the accessory food substances in orange juice would be an adequate substitute for the rice vitamin. Parallel experiments were tried with unpolished rice and malted milk to determine whether or not orange juice would increase the metabolic activities of the organism in media already known to be adequate for growth and maintenance.

The orange juice was prepared by boiling 1 c.c. of orange juice in distilled water and making up the solution to 1 per cent. after evaporation. Previous trial experiments had been made with different percentages of the orange juice, 5 per cent., 1 per cent., 2 per cent., and 3 per cent., and malted milk. The results indicated that the 1 per cent. solution would be most favorable for work with *Paramecium*. The races used for the special problem were isolated from various sources, Pure line A was identical with Pure line 1 of the first experiments, Pure line B and Pure line C were isolated from cultures obtained from Powers and Powers, Lincoln, Neb., and the University of Pennsylvania respectively, Pure line D from a wild culture just introduced into the laboratory, and Pure line E from a mixed culture kept in the laboratory in hay infusion all winter. These gave a variety of physiological conditions upon which to experiment. The individuals in Pure lines B and C were much larger and more vigorous than those from the other lines. The wild race was especially small but very vigorous after the firm establishment of the line, while the races which had been kept in the laboratory all winter were small and sluggish. Following the precautions observed in the previous experiments, four individuals from each line were isolated on April 22, 1918, one in two drops of distilled water and one drop of 1 per cent. malted milk solution, a second in one drop of distilled water and two drops of 1 per cent. malted milk solution, a third in one drop of distilled water, one drop of 1 per cent. malted milk solution and one drop of 1 per cent. orange

juice, and a fourth in two drops of distilled water and one drop of 1 per cent. orange juice.

The work was carried on for eleven days, at the end of which time averages were made from the rates of division in each group. The two control groups showed the influence of different degrees of concentration upon the division rate, and the group in orange juice and water was used to determine whether or not orange juice served alone as an adequate food substance. Results are shown in the accompanying tables.

TABLE IV.

THE DAILY RATE OF DIVISION.

(*a*) 2 drops of distilled water and 1 of malted milk.
(*b*) 1 drop of distilled water and 2 of malted milk.
(*c*) 1 drop of distilled water and 1 of malted milk, and 1 of orange juice.
(*d*) 2 drops of distilled water and 1 of orange juice.

	2d Day.	3d Day.	4th Day.	5th Day.	6th Day.	7th Day.	8th Day.	9th Day.	10th Day.	11th Day.
Line A.										
(*a*).....	2	0	1.5	1	2	1	2	2	1	1.5
(*b*)....	2	1	0	1	1	2	2	1	2	0
(*c*).....	2	2	1	2	1	2	2	2	1	1
(*d*).....	1	0	1	1	dead					
Line B.										
(*a*).....	2	1	2	1	1	2	1	0	1	2
(*b*).....	2	1	1	1	2	1	1	1	1	1
(*c*).....	2	1	1	1	1	2	1	0	1	1
(*d*).....	0	0	dead							
Line C.										
(*a*).....	2	1	1	2	1	1	0	1	1	1
(*b*).....	2	1	1	2	1	2	1	1	1	1
(*c*).....	2	1	2	2	1	1	2	1	1	2
(*d*).....	1	0	0	1	0	dead				
Line D.										
(*a*).....	1	1	2	2	2	1	1	1	1	2
(*b*).....	1	0	2	1	2	2	2	2	1	2
(*c*).....	1	2	0	1	3	2	2	2	1	0
(*d*).....	1	1	1	0	dead					
Line E.										
(*a*).....	1	1	1.5	1.5	0	0	2	2	0	2
(*b*).....	2	1	1.5	1.5	0	1	2	1	1	2
(*c*).....	1	1	2	2	0	1	2	1	0	2
(*d*).....	1	1	1	dead						

The small variations in division rate are within the limits of experimental error, with the exception of group (*d*). From these facts it is to be concluded that orange juice is not an adequate food substance, that it does not increase the metabolic

TABLE V.

TOTAL OF DIVISIONS IN EACH GROUP AND AVERAGES.

Line.	*A.*	*B.*	*C.*	*D.*	*E.*	Total.	10-day Average.	Daily Average.
(*a*).....	14	13	11	14	11	63	12.6	1.26
(*b*).....	12	12	13	15	13	65	13	1.3
(*c*).....	16	11	15	14	12	68	13.6	1.36
(*d*).....	3	0	2	3	3	11	2.2	0.22

activities of organisms in malted milk, that the slight variation in the concentration of media employed in these experiments does not alter the rate of division and finally that these conclusions are applicable to races representing a divergence of previous physiological conditions.

TABLE VI.

THE DAILY RATE OF DIVISION.

(*a*) 2 drops of distilled water and 2 of polished rice water.

(*b*) 2 drops of distilled water and 2 of unpolished rice water.

(*c*) 2 drops of distilled water and 2 of polished rice water and 1 of 1 per cent. orange juice.

(*d*) 2 drops of distilled water and 2 of unpolished rice water and 1 of 1 per cent. orange juice.

	2d Day	3d Day.	4th Day.	5th Day.	6th Day.	7th Day.	8th Day.	9th Day.	10th Day.	11th Day.
Line A.										
(*a*).....	0	0	0	1	0	0	0	dead		
(*b*).....	0	1	1	0	0	1	0	0	0	0
(*c*).....	0	0	0	0	0	0	0	dead		
(*d*).....	1	0	0	0	0	0	0	0	0	0
Line B.										
(*a*).....	0	0	0	0	1	0	0	dead		
(*b*).....	0	2	0	0	1	0	0	0	0	dead
(*c*).....	1	0	0	1	0	0	0	0	0	dead
(*d*).....	1	0	0	1	0	0	0	0	0	0
Line C.										
(*a*).....	0	0	0	0	0	dead				
(*b*).....	0	0	1	0	0	0	dead			
(*c*).....	0	0	0	0	0	0	0	0	dead	
(*d*).....	0	1	0	0	0	0	1	0	dead	
Line D.										
(*a*).....	0	0	0	0	0	dead				
(*b*).....	0	0	1	0	0	dead				
(*c*).....	0	0	0	0	0	0	0	0	0	0
(*d*).....	0	0	0	0	1	0	0	0	0	0
Line E.										
(*a*).....	1	0	0	0	dead					
(*b*).....	0	1	0	0	1	dead				
(*c*).....	1	0	0	1	dead					
(*d*).....	1	0	0	0	1	0	1	0	0	1

TABLE VII.

TOTAL OF DIVISIONS IN EACH GROUP AND AVERAGES.

Line.	*A.*	*B.*	*C.*	*D.*	*E.*	Total.	10-Day Average.	Daily Average.
(*a*).....	1	1	0	0	1	3	0.6	0.06
(*b*).....	3	3	1	1	2	10	2.0	0.2
(*c*).....	0	2	1	1	2	6	1.2	0.12
(*d*).....	1	2	2	1	4	10	2.0	0.2

The experiments with rice and orange juice were begun May 6, by isolating four individuals from Lines A, B, C and D, two as controls in two drops of distilled water and two drops of 1 per cent. rice water, from polished and unpolished rice respectively, and two like the controls with the addition of one drop of 1 per cent. orange juice to each. The work was carried on for eleven days at the end of which time averages were made of the rates of division in each group, to determine whether or not the substitution of the accessory food substances in orange juice for the rice vitamin had increased the metabolic activities of the individuals in polished rice, and also to note any stimulus to division produced by the addition of orange juice to unpolished rice. The results are shown in the accompanying tables.

It is evident from these results that all of the *Paramecia* were in a state of depression which was not altered to any appreciable extent by the presence of orange juice, although the individuals in unpolished rice and orange juice seem to have had more resistance to the unknown factors which produced dissolution in the other groups. The orange juice had a slightly stimulating effect upon the metabolic activities of the organisms in polished rice but absolutely no effect whatever upon the division rate of those in unpolished rice. From these results it may be concluded that orange juice has not a powerful influence upon the metabolic activities of *Paramecium*.

Much appreciation is due Dr. Florence Peebles for her helpful supervision of this work.

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GLYCOGEN IN THE CHICK EMBRYO.

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In his paper, "Glycogen in the Nervous System of Vertebrates"[1] Gage has pointed out that glycogen is not found in such great abundance in the organs of the chick at any one period of its development as in the embryonic organs of many other forms, including mammals; from which the inference is at once drawn that this difference is correlated with the nature of development in the hen's egg, where the yolk furnishes an abundant supply of food, available at any moment for the needs of the embryo. The development of the various organs is a gradual one as compared with that of forms which are provided with less yolk, and glycogen, the builder of tissue and energy producer, appears only as it is needed in the successive stages.

This is particularly striking when comparing the chick with such forms as *Petromyzon* and *Amblystoma*, studied by Gage. When contrasted with mammals the same explanation is not as valid, for the mammalian tissue is also able to develop gradually, calling upon the maternal supply when necessary. However it may be supposed that in mammals the embryo is less directly in contact with its food supply than in the chicken. A great amount of glycogen could not be produced as quickly when a demand arose in tissue development, and consequently a greater supply is produced and maintained in the embryonic tissue itself.

The following observations in regard to the occurrence and distribution of glycogen in the tissue of the chick, from primitive streak stages to the age of about ten days, are based on material which was fixed and stained according to the methods given in Gage's article, and in all cases the presence of the glycogen has been verified by testing the sections with saliva, according to Gage's directions.

It gives me pleasure to be able to acknowledge my great

[1] *Jour. Comp. Neur.*, Vol. 27, No. 4.

indebtedness to Dr. H. L. Wieman, under whose direction this problem was undertaken, and whose assistance, interest and criticism made it possible to carry out the work.

Perhaps the most striking fact observed in the formation of glycogen in the chick was its almost invariable presence in the yolk-sac, from the earliest stages to chicks of ten days. Fig. 1 (Plate I.) shows globules of glycogen in the yolk-sac of a chick of six somites, before the vascular area has developed. In these earlier stages the glycogen is located in the yolk-sac near the embryo, and it is found more or less consistently distributed throughout the vascular area when this has been formed, more abundant near the embryo than towards the edges. It gives to the walls of the blood-vessels of this region a striking red color and under high power appears as mahogany-colored masses scattered among the cells. Fig. 2 shows a part of the vascular area of a six-day chick with the glycogenated areas represented in stippling in the walls of the blood-vessels. Figs. 2*a* and 2*b* show parts of the vascular areas of seventy-two-hour and four-day chicks respectively, under higher magnification. In Fig. 2*b* the actual globules of glycogen are represented in black. Apparently the yolk-sac furnishes a way-station in which carbohydrates are stored as glycogen on their way from the yolk to the embryonic tissue.

This formation of glycogen in the yolk-sac makes an interesting comparison with the formation of glycogen in the placenta of mammals, a fact which seems well established. In mammals the maternal glycogen-forming tissues are perhaps relieved in in this way of the strain of a sudden call for large amounts of sugar. In both cases a supply of glycogen is on hand ready-formed and able to be called upon quickly.

In the earliest chick embryos observed there appeared to be, here and there, a trace of glycogen in the yolk itself in the form of globules somewhere near the site of the embryo, but this fact is not at present established beyond a doubt in my mind, although it would accord with what Gage has observed in the eggs of the *Petromyzon* and *Amblystoma*.

In embryos of from fifteen to twenty-four hours glycogen was found in the ectoderm of the head region, both in the developing

nervous system and in the ectoderm below the headfold (Fig. 3).

As soon as the heart tissue begins to develop (at about twenty-eight hours) it takes on its glycogenic function and from that time to the oldest embryos studied the heart is the most consistently and by far the most abundantly glycogenated organ of the body. The glycogen here appears in very deeply stained granules embedded close together throughout the muscular walls of the entire heart, and extending into the sinus venosus and the bulbus. Fig. 4 shows, in stippling, the position of the glycogen in the heart tubes of a chick of nine somites. Fig. 5 shows a part of the heart of a six-day chick, with glycogen abundant in the muscles. Fig. 5*a* represents a part of the wall of a four-day chick's heart, and Fig. 5*b* of a ten-day chick's heart, under high power. In both of these the actual globules of glycogen have been represented in black.

The glycogen of the vascular area seems to be continued under the embryo with more or less regularity. Certainly as the gut is formed glycogen extends into it, appearing in some cases very distinctly at the opening of the gut onto the yolk, more abundant at the opening of the hind-gut than of the foregut, extending in a thirty-five-hour chick into the pharynx, and from fifty-six hours on being very noticeable in the intestine. Fig. 6 shows the fore-gut of a four-and-a-half-day chick just in front of the anterior portal. The glycogen in the entoderm of the gut and below it is stippled. Fig. 7 is the posterior intestinal portal of a seven-day chick, and Fig. 7*a* is the intestine and splanchnic entoderm of the same a few sections posterior to Fig. 7. Fig. 8 is a transverse section through the intestine of a ten-day chick, showing glycogen abundant in the epithelium.

At fifty-six hours, and in all later stages, glycogen was found in the myotomes, being particularly striking in those sections where the body was cut sagitally, the glycogen here appearing like mahogany-colored beads on the spindle-shaped myotomes (Fig. 10, Plate II.). Fig. 9 (Plate II.) is a transverse section of a chick of five days showing the position of the glycogen in the myotomes. Under high power one of these myotomes appeared as in Fig. 11, where the actual spots of glycogen are represented. As other muscular parts develop the myotomes become less

glycogenated, but they still retain this function to some extent.

In a seventy-two-hour chick there was an appearance of glycogen in the mesoderm between the two portals and the two sides of the future body-cavity, but as this did not seem consistent with other stages, its presence, or the normality of its presence, is rather doubtful.

In a ninety-six-hour chick a faint trace of glycogen was found in the septum medullæ. At four and a half days the glycogen was very distinct here and it remained in this position up to the oldest stage studied, making a very striking mahogany-colored streak along the floor of the medulla (Figs. 12 and 13). From five days on it was found in a similar position along the floor of the spinal cord in its lumbar and sacral regions (Fig. 14).

At this same age (five days), it appeared in the ectoderm of the anal plate.

At approximately six days glycogen appeared in some of the muscular tissue around the eyes, forming strings of beads along the fibers of the tissue. This became more and more abundant in later stages (Figs. 15 and 16).

At an age of from seven to eight days it was observed in other parts of the head region—in the muscular tissue below the tongue, and medially in a clearly defined area of developing cartilage which formed a keel-like structure below the brain and between it and the pharynx. (Fig. 17).

At this age also glycogen occurs in the muscular tissue along the sides of the body, and at a slightly older stage it appears in a small patch of ectoderm in the nose region.

At ten days it is found in the cartilage of the head and body quite generally. Fig. 18 shows a trace of it in a developing centrum in the head region. In Fig. 14 it appears in the vertebral cartilage of the body. Fig. 19 shows it very abundant in the cartilage of one of the developing limbs. Fig. 19*a* shows the same under high power. The glycogen of the cartilage appears as very distinct red spherules which practically fill the cells.

Glycogen is found in great abundance at this age in the muscular tissue, not only in that already mentioned, but also in the more superficial muscles of the head, in the pectoral muscles (Fig. 20), in the muscles of the limbs (Fig. 19), and in fact in

practically all of the musculature of the body. In some places it seemed to occur in the deeper layer of the skin.

It appeared at this age also in a mass of nerve cells lying in the dorsal fissure of the spinal cord in its lumbar region (Fig. 14). Its occurrence in these cells was also noted by Gage.

These results are perhaps more significant in a negative than a positive way. They show that in the chick the storage of glycogen in the embryonic tissue itself is not as extensive as in other forms, and that it does not become a well-developed function of the embryo in its earliest stages, though glycogen does appear in some of the tissue and becomes more and more abundant in later stages. The embryo depends for its energy production and building material upon the food of the yolk, a part of which is kept on hand in the form of glycogen in the yolk-sac.

The majority of the drawings were made with a 16 mm. objective and 4x eye-piece. For those marked high power a 4 mm. objective, 4x eye-piece were used. All drawings were made with a camera lucida.

Explanation of Plate I.

Fig. 1. Yolk-sac of a six-somite chick before development of the vascular area. Glycogenated cells in black.

Fig. 2. Splanchnopleure of the vascular area of a six-day chick with glycogen in the walls of the blood-vessels (stippled). *bl. v.*, blood-vessels; *cæl.*, cœlome; *y.s.*, yolk-sac.

Fig. 2*a*. Vascular area of a seventy-two-hour chick under high power. (Glycogen stippled.)

Fig. 2*b*. Vascular area of a four-day chick, high power. Actual masses of glycogen represented in black.

Fig. 3. Glycogen (stippled) in the ectoderm of a chick with six somites. *ect.*, ectoderm.

Fig. 4. Glycogen (stippled) in the heart tubes of a chick with nine somites. *ht.*, heart; *ph.*, pharynx.

Fig. 5. Heart of a six-day chick with glycogen abundant in the muscular walls. (Stippled.) *l.a.*, left auricle; *l.v.*, left ventricle; *r.a.*, right auricle; *r.v.*, right ventricle.

Fig. 5*a*. Wall of a four-day chick's heart under high power. The black portions represent glycogen.

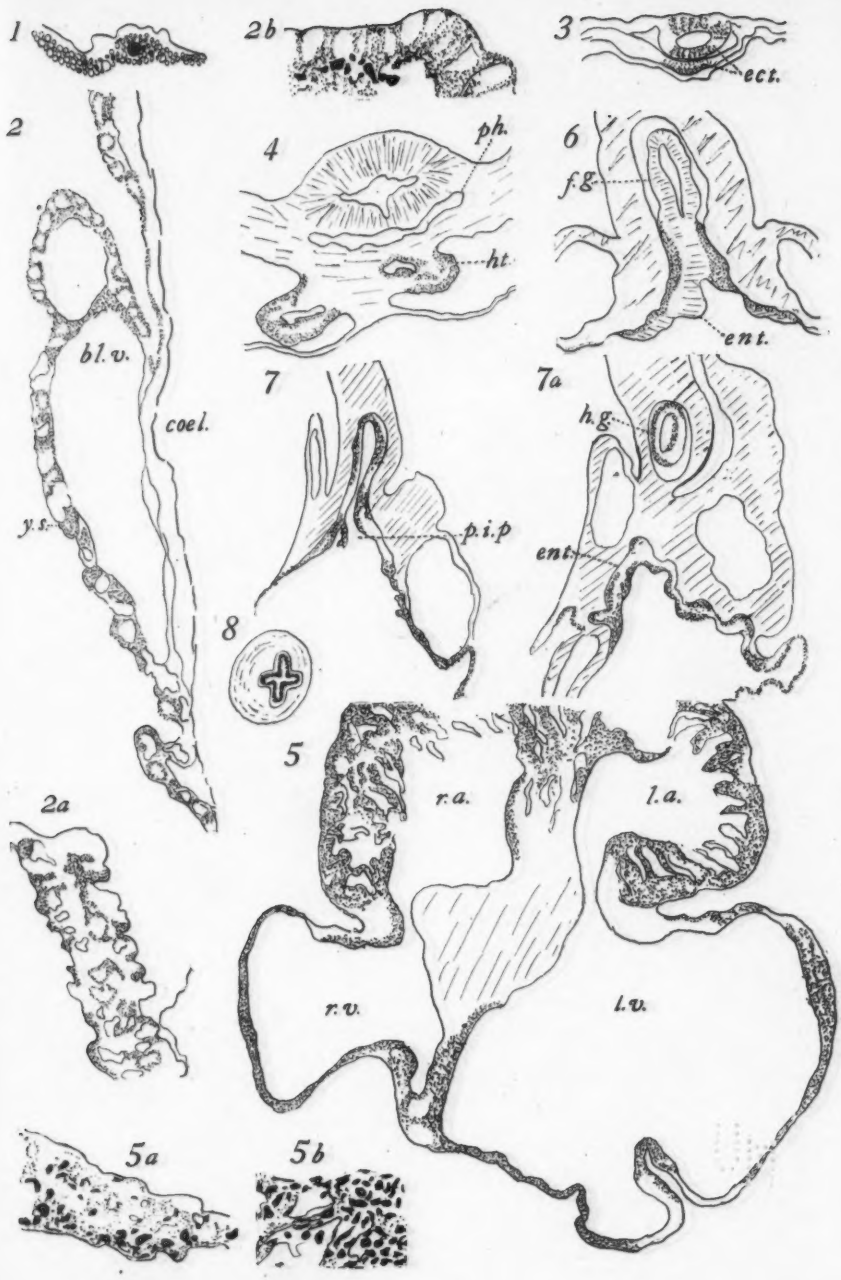
Fig. 5*b*. Ten-day chick's heart. High power. Globules of glycogen in black.

Fig. 6. Fore-gut of a four-and-a-half-day chick just anterior to the anterior portal. (Glycogen stippled.) *ent.*, entoderm; *f. g.*, fore-gut.

Fig. 7. Posterior intestinal portal of a seven-day chick. (Glycogen stippled.) *p.i.p.*, posterior intestinal portal.

Fig. 7*a*. Hind-gut and splanchnic entoderm of the same, a few sections posterior to 7. (Glycogen stippled.) *ent.*, entoderm; *h.g.*, hind-gut.

Fig. 8. Cross section through intestine of a ten-day chick. (Glycogen stippled.)



H. JEANETTE ALLEN.

Explanation of Plate II.

Fig. 9. Transverse section of a five-day chick showing glycogen (stippled) in the myotomes and intestine. *m.y.*, myotomes; *int.*, intestine

Fig. 10. Sagittal section through the posterior end of a seven-day chick showing glycogen (stippled and dotted) in the myotomes. *m.y.*, myotomes.

Fig. 11. A myotome in sagittal section. Five days. High power. The black areas are glycogen.

Fig. 12. Transverse section of the medulla oblongata of a six-day chick. (Glycogen stippled.) *m.*, medulla; *s.m.*, septum medullæ.

Fig. 13. Sagittal section of the septum medullae. Six to seven days. (Glycogen stippled.) *c.*, cord, *s.m.*, septum medullæ.

Fig. 14. Transverse section of spinal cord (lumbar region) of a ten-day chick, showing glycogen (stippled) in the cartilage of the vertebra, the floor of the cord and a mass of nerve cells in the dorsal fissure. *ca.*, cartilage; *n.*, glycogenated nerve tissue.

Fig. 15. Glycogenated muscle near the eye of a six-day chick. (Glycogen stippled.) *dien.*, diencephalon; *no.*, optic nerve; *o.*, eye.

Fig. 16. Glycogenated muscles near the eyes of a seven-day chick. (Abbreviations as in 15.)

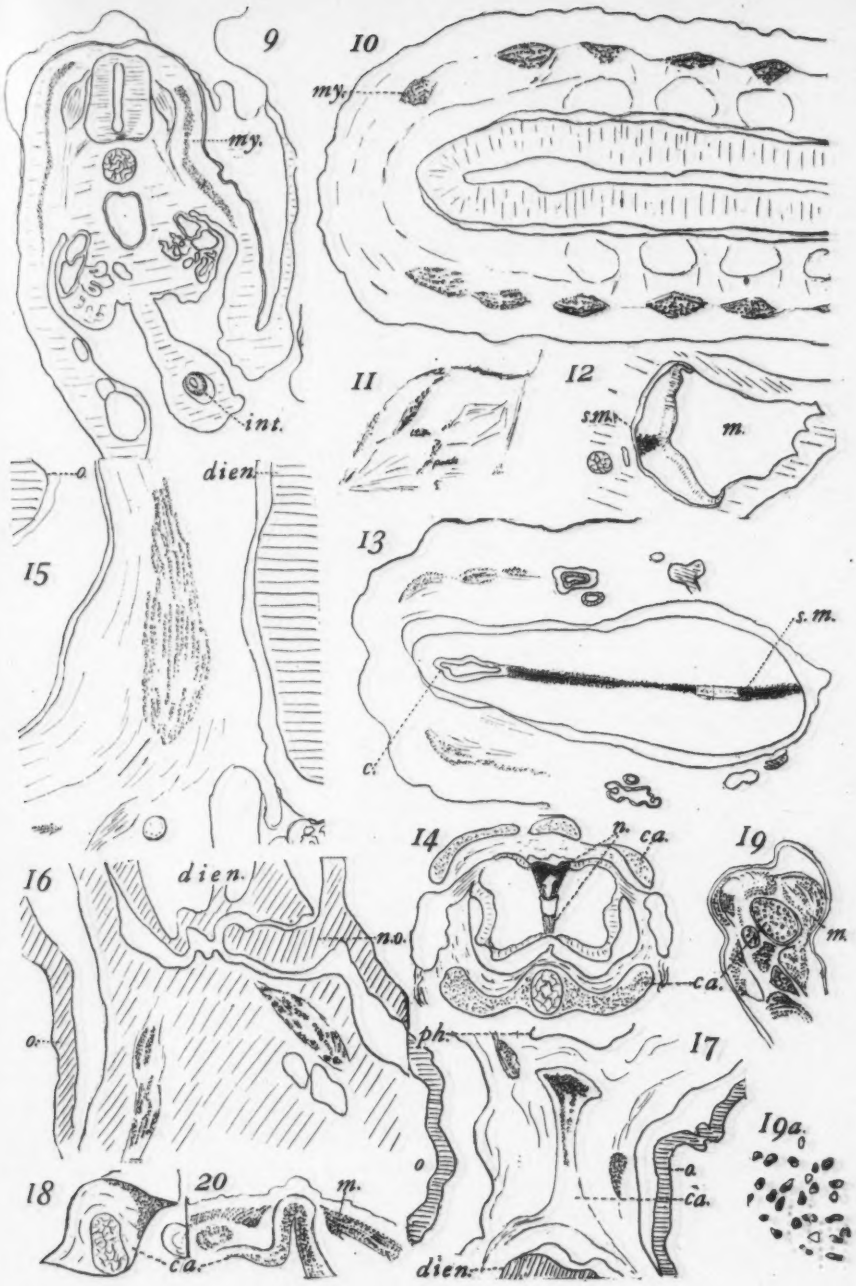
Fig. 17. Glycogenated cartilage of developing skull. Seven to eight days *ca.*, cartilage; *dien.*, diencephalon; *o.*, eye; *ph.*, pharynx.

Fig. 18. Developing centrum in the head of a ten-day chick, showing glycogenated area in stippling. *ca.*, cartilage.

Fig. 19. Glycogenated cartilage and muscles of the foot of a ten-day chick. (Glycogen stippled and dotted.) *ca.*, cartilage; *m.*, muscles.

Fig. 19a. Cartilage of the same under high power.

Fig. 20. Pectoral muscles and cartilage of a ten-day chick.



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